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Functional characterization of human and cynomolgus monkey cytochrome P450 2E1 enzymes

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Abstract

Cytochrome P450 2E1 (CYP2E1) is an enzyme of major toxicological interest because it metabolizes various drugs, precarcinogens and solvents to reactive metabolites. In this study, human and cynomolgus monkey CYP2E1 cDNAs (humCYP2E1 and monCYP2E1, respectively) were cloned, and the corresponding proteins were heterologously expressed in yeast cells to identify the functions of primate CYP2E1s. The enzymatic properties of CYP2E1 proteins were characterized by kinetic analysis of chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation. humCYP2E1 and monCYP2E1 enzymes showed 94.3% identity in their amino acid sequences. The functional CYP content in yeast cell microsomes expressing humCYP2E1 was 38.4 pmol/mg protein. The level of monCYP2E1 was 42.7% of that of humCYP2E1, although no significant differences were statistically observed. The K_m values of microsomes from human livers and yeast cells expressing humCYP2E1 for CYP2E1-dependent oxidation were 822 and 627 μM for chlorzoxazone 6-hydroxylation, and 422 and 514 μM for 4-nitrophenol 2-hydroxylation, respectively. The K_m values of microsomes from cynomolgus monkey livers and yeast cells expressing monCYP2E1 were not significantly different from those of humans in any enzyme source. V_{max} and V_{max}/K_m values of human liver microsomes for CYP2E1-dependent oxidation were 909 pmol/min/mg protein and 1250 nl/min/mg protein for chlorzoxazone 6-hydroxylation, and 1250 pmol/min/mg protein and 2990 nl/min/mg protein for 4-nitrophenol 2-hydroxylation, respectively. The kinetic parameter values of cynomolgus monkey livers were comparable to or lower than those of human liver microsomes (49.5–102%). In yeast cell microsomes expressing humCYP2E1, V_{max} and V_{max}/K_m values for CYP2E1-dependent oxidation on the basis of CYP holoprotein level were 170 pmol/min/pmol CYP and 272 nl/min/pmol CYP for chlorzoxazone 6-hydroxylation, and 139 pmol/min/pmol CYP and 277 nl/min/pmol CYP for 4-nitrophenol 2-hydroxylation, respectively, and the kinetic parameters of monCYP2E1 exhibited similar values. These findings suggest that human and cynomolgus monkey CYP2E1 enzymes have high homology in their amino acid sequences, and that their enzymatic properties are considerably similar. The information gained in this study should help with in vivo extrapolation and to assess the toxicity of xenobiotics.
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Introduction

Cytochrome P450 (CYP) enzymes are hemoproteins that are responsible for the oxidative metabolism of exogenous chemicals such as drugs and environmental chemicals, as well as endogenous substances such as steroids and fatty acids (Gonzalez, 1990; Rendic and Di Carlo, 1997). They comprise a

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superfamily of related enzymes that are grouped into families and subfamilies based on similarities in amino acid or nucleotide sequences (Nelson et al., 1996). Most of the exogenous chemicals have been shown to be oxidized mainly by CYP1–3 families, and individual CYP isoforms have considerable, but overlapping, substrate specificities (Gonzalez, 1990; Nelson et al., 1996; Rendic and Di Carlo, 1997).

The human *CYP2E1* gene is located in the 10q24.3-qter region of chromosome 10, and spans 11,413 base pairs with nine exons, eight introns and typical TATA box (Umeno et al., 1988; Nelson et al., 1996). CYP2E1 enzyme consists of 493 amino acid residues and is expressed mainly in the liver (Ingelman-Sundberg et al., 1993; Nelson et al., 1996). CYP2E1 as well as other isoforms of CYP1–3 families has broad substrate specificity; more than 80 low-molecular hydrophobic chemicals have been identified as substrates for this CYP isoform (Guengerich et al., 1991; Rendic and Di Carlo, 1997). CYP2E1 has also been reported to activate many environmental chemicals such as benzene, toluene and *N*-nitrosamines, and several drugs such as acetaminophen and chlorzoxazone to highly reactive metabolites (Guengerich et al., 1991; Lieber, 1997). CYP2E1 activity is mediated by various pathophysiological conditions such as diabetes, obesity and starvation, and also by a number of environmental factors (Gonzalez, 1990; Lucas et al., 1998; McCarver et al., 1998). The expression levels of hepatic CYP2E1 vary by orders of magnitude in humans and are found to be elevated in alcoholics (Perrot et al., 1989; Ingelman-Sundberg et al., 1993). Animal studies of CYP2E1 regulation have been found to be very complex, involving aspects of developmental and hormonal regulation, transcriptional and posttranscriptional control, and protein stabilization (Gonzalez, 1990; Yang et al., 1990; Ingelman-Sundberg et al., 1993). Furthermore, it has been suggested that there are large inter-individual variations in the protein level and catalytic activity of CYP2E1 in human liver microsomes (Yoo et al., 1988; Shimada et al., 1994). Previous in vivo studies of a Caucasian population using chlorzoxazone as a probe drug reported a several-fold variation in clearance with a normal distribution of activities, and suggested that such variation is caused in part by the genetic polymorphism of *CYP2E1* (Kim and O'Shea, 1995; Lucas et al., 1995).

The CYP2E subfamily has been shown to have only a single isoform in most mammals, and CYP2E1 cDNAs of humans, monkeys, rabbits, rats and mice were cloned (Nelson et al., 1996). In general, nonhuman primates such as rhesus monkeys (*Macaca mulatta*) and cynomolgus monkeys (*Macaca fascicularis*) are regarded as experimental animals closer to humans in studies on safety evaluation and biotransformation for drug development. Rhesus monkey CYP2E1 cDNA has been cloned (GenBank accession number, AY635465); however, the functions of protein encoded by cDNA have not been characterized. Although cynomolgus monkey CYP2E1 cDNA, which is presumed to lack N-terminal 45 residues as compared with the sequence for human CYP2E1, has been isolated from a liver DNA library (Komori et al., 1992; GenBank accession number, S55205), the complete cDNA has not been cloned yet. Furthermore, examination of the functional characterization of monkey CYP2E1 enzymes is an important aspect of drug metabolism research.

Table 1
Primers used for construction of OR, humCYP2E1 and monCYP2E1 expression plasmids

Primer name	Sequence
OR-F	5′-CCCAAGCTTGGGAA*AAAA*ATGGGAGACTCC-3′
OR-R	5′-GGGGTACCCCCTAGCTCCACACGTCC-3′
humCYP2E1-F	5′-AAGCTTAA*AAAA*ATGTCTGCCCTCGGAGTG-3′
humCYP2E1-R	5′-AAGCTTTCATGAGCGGGGAATGACACA-3′
monCYP2E1-F1	5′-TTCCTTTCCACAGGATTGTCCTCCCGG-3′
monCYP2E1-R1	5′-GATTCTGGGGGTCCTCCTCACACTCTA-3′
monCYP2E1-F2	5′-AAGCTTAA*AAAA*ATGTCTGCCCTCGGAGTC-3′
monCYP2E1-R2	5′-AAGCTTTCATGAACGGGGAATGACACAG-3′

Underlined letters indicate restriction enzyme sites. Italic letters indicate the yeast consensus sequence.

The purpose of this study was to examine the similarities and differences in enzymatic properties of CYP2E1 between humans and cynomolgus monkeys. To achieve this, human and cynomolgus monkey CYP2E1 cDNAs were cloned, and the corresponding CYP2E1 proteins were heterologously expressed in yeast cells. The enzymatic properties of CYP2E1 proteins were examined by kinetic analysis using chlorzoxazone and 4-nitrophenol as substrates.

Materials and methods

Materials

Three individual human liver microsomes (one man, 77 years old; two women, 54 and 56 years old) were purchased from BD Biosciences (San Jose, CA). Three male cynomolgus monkey livers (4 years old, 2.7–2.9 kg) were supplied by Ina Research Inc. (Ina, Japan). The use of human and cynomolgus monkey livers for this study was approved by the ethics review board of Okayama University. The RNeasy mini kit, QIAshredder and MinElute Gel Extraction kit were purchased from Qiagen (Hilden, Germany); the RNA PCR kit v3.0 and *Hin*dIII were from Takara Bio (Ohtsu, Japan); Taq DNA polymerase and pGEM-T vector were from Promega (Madison, WI); KOD-plus DNA polymerase was from Toyobo (Osaka, Japan); BigDye terminator cycle sequencing reaction kit v3.1 was from Applied Biosystems (Foster City, CA); pYES3/CT and pYES2/CT yeast expression vectors, and INV*Sc*1 yeast strain were from Invitrogen (Carlsbad, CA); yeast nitrogen base was from BD Diagnostics (Franklin Lakes, NJ); Zymolyase 100T was from Seikagaku Corporation (Tokyo, Japan); cytochrome *c* from horse heart and chlorzoxazone were from Sigma-Aldrich (St. Louis, MO); 6-hydroxychlorzoxazone was from Daiichi Pure Chemicals (Tokyo, Japan); 4-nitrophenol, 4-nitrocatechol and salicylamide were from Nacalai Tesque (Kyoto, Japan); NADPH, $NADP^+$, glucose 6-phosphate and glucose 6-phosphate dehydrogenase were from Oriental Yeast (Tokyo, Japan); rabbit anti-human CYP2E1 antibody was from Novus Biologicals (Littleton, CO); peroxidase-conjugated goat anti-rabbit immunoglobulin was from Zymed Laboratories (South San Francisco, CA); and enhanced chemiluminescence-plus

reagents were from GE Healthcare Bio-Sciences (Little Chalfont, UK). All other chemicals and reagents used were of the highest quality commercially available.

Construction of OR, humCYP2E1 and monCYP2E1 expression plasmids

Full-length cDNAs encoding human NADPH-cytochrome P450 reductase (OR), human CYP2E1 (humCYP2E1) and cynomolgus monkey CYP2E1 (monCYP2E1) were amplified by polymerase chain reaction (PCR) using the forward and reverse primers listed in Table 1. The restriction enzyme sites (*Hin*dIII for OR-F, humCYP2E1-F, humCYP2E1-R, monCYP2E1-F2 and monCYP2E1-R2, and *Kpn*I for OR-R) were introduced to the 5′-end of the start codon and the 3′-end of the stop codon to facilitate subcloning into pYES3/CT or pYES2/CT expression vector. Yeast consensus sequences (Romanos et al., 1992) were also introduced upstream of the start codon in OR-F, humCYP2E1-F and monCYP2E1-F2 to achieve a high expression of protein in yeast cells.

To achieve the stable expression of OR in yeast cells, pYES3/CT expression vector was used. The full-length cDNA encoding OR was amplified by PCR from OR cDNA cloned into pGEM-3Z vector (Narimatsu et al., 2006) as a template using OR-F and OR-R primer pair. The PCR reaction mix contained 1×PCR buffer, 0.2 mM dNTPs, each primer at 0.5 μM and 1 U KOD-plus DNA polymerase in a final volume of 50 μl. PCR consisted of 28 cycles with denaturation at 94 °C for 15 s, annealing at 58 °C for 30 s and extension at 68 °C for 150 s. Initial denaturation and final extension were at 94 °C for 120 s and at 68 °C for 420 s, respectively. The resulting PCR product was isolated by electrophoresis on a 1.0% TAE agarose gel and gel-purified with a MinElute Gel Extraction kit, and A-tailing of the 3′-flanking region was performed using Taq DNA polymerase. The PCR product was directly introduced into pGEM-T vector using the TA cloning system. The plasmid was sequenced in both forward and reverse directions using a BigDye terminator cycle sequencing reaction kit v3.1 to confirm that there were no PCR errors. DNA fragment corresponding to OR was cut from the pGEM-T plasmid with *Hin*dIII and *Kpn*I, and subsequently subcloned into the pYES3/CT expression vector digested with *Hin*dIII and *Kpn*I (pYES3/OR). The expression plasmid was sequenced to verify the correct orientation with respect to the promoter for pYES3/CT. *Saccharomyces cerevisiae* INV*Sc*1 strain was transformed with the plasmid pYES3/OR by the lithium acetate procedure (Schiestl and Gietz, 1989), and the transformant (INV*Sc*1/OR) was selected using *TRP1* auxotrophic marker.

The full-length cDNA encoding humCYP2E1 was amplified by PCR from humCYP2E1 cDNA cloned into pENTR/D-TOPO vector (Hanioka et al., 2003) as a template using humCYP2E1-F and humCYP2E1-R primer pair. The PCR reaction mix contained 1×PCR buffer, 0.2 mM dNTPs, each primer at 0.5 μM and 1 U KOD-plus DNA polymerase in a final volume of 50 μl. PCR consisted of 28 cycles with denaturation at 94 °C for 15 s, annealing at 60 °C for 30 s and extension at 68 °C for 90 s. Initial denaturation and final extension were at 94 °C for 120 s and at 68 °C for 300 s, respectively. The resulting PCR product was isolated and purified by agarose electrophoresis. The PCR product was introduced into the pGEM-T vector after A-tailing and sequenced in both forward and reverse directions. A DNA fragment corresponding to humCYP2E1 was cut from the pGEM-T plasmid with *Hin*dIII, and subsequently subcloned into the pYES2/CT expression vector digested with *Hin*dIII (pYES2/humCYP2E1). The expression plasmids were sequenced to verify the correct orientation with respect to the promoter for pYES2/CT.

Total RNA was extracted from cynomolgus monkey liver using an RNeasy mini kit and QIAshredder according to the manufacturer's instructions. The total RNA was reverse-transcribed to cDNA using RNA PCR kit v3.0. The full-length cDNA encoding monCYP2E1 was amplified by PCR from single-strand cDNA templates using a monCYP2E1-F1 and monCYP2E1-R1 primer pair. These primers were designed based on the nucleotide sequence in the flanking regions of humCYP2E1 cDNA (GenBank accession number, J02843). The PCR reaction mix contained 1×PCR buffer, 0.2 mM dNTPs, each primer at 0.5 μM and 1 U KOD-plus DNA polymerase in a final volume of 50 μl. PCR consisted of 35 cycles with denaturation at 94 °C for 15 s, annealing at 61 °C for 30 s and extension at 68 °C for 90 s. Initial denaturation and final extension were at 94 °C for 120 s and at 68 °C for 300 s, respectively. The resulting PCR product was isolated and purified by agarose electrophoresis, and the 5′- and 3′-ends of the coding region were sequenced in both the forward and reverse directions. The full-length cDNA thus obtained was modified by PCR amplification with monCYP2E1-F2 and monCYP2E1-R2 as primers. The PCR reaction mix contained 1×PCR buffer, 0.2 mM dNTPs, each primer at 0.5 μM and 1 U KOD-plus DNA polymerase in a final volume of 50 μl. PCR consisted of 30 cycles with denaturation at 94 °C for 15 s, annealing at 57 °C for 30 s and extension at 68 °C for 90 s. Initial denaturation and final extension were at 94 °C for 120 s and at 68 °C for 300 s, respectively. The resulting PCR product was isolated and purified by agarose electrophoresis. The PCR product was introduced into the pGEM-T vector after A-tailing and sequenced in both forward and reverse directions. A DNA fragment corresponding to monCYP2E1 was cut from the pGEM-T plasmid with *Hin*dIII, and subsequently subcloned into pYES2/CT expression vector digested with *Hin*dIII (pYES2/monCYP2E1). The expression plasmids were sequenced to verify the correct orientation with respect to the promoter for pYES2/CT.

Expression of humCYP2E1 and monCYP2E1 enzymes

The transformant INV*Sc*1/OR was transformed with the expression plasmid containing humCYP2E1 or monCYP2E1 by the lithium acetate procedure (Schiestl and Gietz, 1989). *TRP1* and *URA3* auxotrophic markers were used to select these transformants. For the production of CYP2E1 proteins, yeast cells were grown at 30 °C with shaking to an OD_{600} of 4.0 in 200 ml of selective medium containing 2% glucose as the carbon source. Cells corresponding to 0.4 OD_{600} were

inoculated into 2000 ml of induction medium containing 2% galactose and 1% raffinose as the carbon source. After cultivation at 30 °C for 24 h, microsomal fractions from yeast cells were prepared by the method of Oeda et al. (1985) with some modifications. The cells were harvested by centrifugation at 5000 *g* for 10 min at 4 °C, washed with 400 ml of water and centrifuged at 5000 *g* for 10 min at 4 °C. The pellets were suspended in 200 ml of buffer A (20 mM Tris–HCl (pH 7.4) containing 2 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA and 50 U/ml Zymolyase 100T), and incubated at 35 °C for 2 h with gentle shaking. The pellets obtained after centrifugation at 5000 *g* for 20 min at 4 °C were washed again with 200 ml of buffer A before being resuspended in 100 ml of buffer B (20 mM Tris–HCl (pH 7.4) containing 0.6 M sorbitol, 0.1 mM dithiothreitol, 0.1 mM EDTA and 0.5 mM phenylmethylsulfonyl fluoride), and sonicated on ice 10 times (30 s each time). For

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   1 ATGTCTGCCCTCGGAGTCAGCGTGGCCCTGTTGGTGTGGGTGGCCGTCCTCCTGCTGGTG 60
   1  M  S  A  L  G  V  S  V  A  L  L  V  W  V  A  V  L  L  L  V 20

  61 TCCATCTGGAGGCAGGTGCACAGCAGCTGGAATCTGCCCCCAGGCCCTTTCCCACTTCCC 120
  21  S  I  W  R  Q  V  H  S  S  W  N  L  P  P  G  P  F  P  L  P 40

 121 ATCATCGGGAACCTCTTCCAGTTGGAATGGAAGAATATTCCCAAGTCCTTCACCCGGCTG 180
  41  I  I  G  N  L  F  Q  L  E  W  K  N  I  P  K  S  F  T  R  L 60

 181 GCCCAGCGCTTCGGGCCGGTGTTCACGCTGTACGTGGGCTCGCGGCGCGTGGTGGTTGTG 240
  61  A  Q  R  F  G  P  V  F  T  L  Y  V  G  S  R  R  V  V  V  V 80

 241 CACGGCTACAAGGCGGTGAGGGAAGTGCTGCTGGACCACAAGGACGAGTTCTCGGGCAGA 300
  81  H  G  Y  K  A  V  R  E  V  L  L  D  H  K  D  E  F  S  G  R 100

 301 GGCGACATCCCCGCGTTCCACGCGCACAGGGACAGGGGAATTATTTTCAATAACGGACCT 360
 101  G  D  I  P  A  F  H  A  H  R  D  R  G  I  I  F  N  N  G  P 120

 361 ACCTGGAAGGACACCCGGCGGTTTTCCCTGACCACCCTCCGGAACTATGGGATGGGGAAA 420
 121  T  W  K  D  T  R  R  F  S  L  T  T  L  R  N  Y  G  M  G  K 140

 421 CAGGGCAACGAGAGCCGGATCCAGAGGGAGGCCCACTTCCTGCTGGAAGCGCTCAGGAAG 480
 141  Q  G  N  E  S  R  I  Q  R  E  A  H  F  L  L  E  A  L  R  K 160

 481 ACCCAAGGCCAGCCTTTCGACCCCACCTTCCTCATCGGCTGTGCGCCCTGCAACGTCATA 540
 161  T  Q  G  Q  P  F  D  P  T  F  L  I  G  C  A  P  C  N  V  I 180

 541 GCCGACATCCTCTTCCGCAAGCATTTTGACTACAATGATGAGAAGTTTCTGAGGCTGATG 600
 181  A  D  I  L  F  R  K  H  F  D  Y  N  D  E  K  F  L  R  L  M 200

 601 TATTTGTTTAATGAGAACTTCCAGCTGCTCAGCACTCCCTGGCTCCAGCTTTACAATAAT 660
 201  Y  L  F  N  E  N  F  Q  L  L  S  T  P  W  L  Q  L  Y  N  N 220

 661 TTCCCCAGCTTACTACACTACTTGCCTGGAAGCCATAGAAAAGTCATGAAAAATGTGGCT 720
 221  F  P  S  L  L  H  Y  L  P  G  S  H  R  K  V  M  K  N  V  A 240

 721 GAAATAAAGGAGTATGTGTCTGAAAGGGTGAAGGAGCACCTTCAATCTCTGGACCCCAAC 780
 241  E  I  K  E  Y  V  S  E  R  V  K  E  H  L  Q  S  L  D  P  N 260

 781 TGCCCCCGGGATCTCACCGACTGCCTGCTTGTGGAAATGGAGAAGGAAAAGCACAGTGCA 840
 261  C  P  R  D  L  T  D  C  L  L  V  E  M  E  K  E  K  H  S  A 280

 841 GAGCGCTTGTACACAATGGACGGTATCACCGTGACTGTGGCCGACCTGTTCTTTGCGGGG 900
 281  E  R  L  Y  T  M  D  G  I  T  V  T  V  A  D  L  F  F  A  G 300

 901 ACAGAGACCACCAGCACCACTCTGAGATATGGGCTCCTGATTCTCATGAAATACCCTGAG 960
 301  T  E  T  T  S  T  T  L  R  Y  G  L  L  I  L  M  K  Y  P  E 320

 961 ATCGAAGAGAAGCTCCATGAAGAAATTGACAGGGTGATTGGGCCAAGCCGAATCCCCGCC 1020
 321  I  E  E  K  L  H  E  E  I  D  R  V  I  G  P  S  R  I  P  A 340

1021 ATCAAGGATAGGCAAGAGATGCCCTATATGGATGCTGTGGTGCATGAGATTCAGCGGTTC 1080
 340  I  K  D  R  Q  E  M  P  Y  M  D  A  V  V  H  E  I  Q  R  F 360

1081 ATCACCCTCGTGCCCTCCAACCTGCCCCATGAAGCAACCCGAGACACCATTTTCAGAGGA 1140
 361  I  T  L  V  P  S  N  L  P  H  E  A  T  R  D  T  I  F  R  G 380

1140 TACATCATCCCCAAGGGCACGGTCATAGTGCCAACTCTGGACTCTGTTTTGTATGACAAC 1200
 381  Y  I  I  P  K  G  T  V  I  V  P  T  L  D  S  V  L  Y  D  N 400

1201 CAAGAATTTCCTGATCCAGAAAAGTTTAAGCCAGAACACTTCCTGGATGAAAGTGGGAAG 1260
 401  Q  E  F  P  D  P  E  K  F  K  P  E  H  F  L  D  E  S  G  K 420

1261 TTCAAGTACAGTGACTATTTCAAGCCATTTTCCGCAGGAAAACGAGTGTGTGCTGGAGAA 1320
 421  F  K  Y  S  D  Y  F  K  P  F  S  A  G  K  R  V  C  A  G  E 440

1321 GGCTTGGCTCGCATGGAGTTGTTTCTATTGTTGTCTGCCATTTTGCAGCATTTTAATTTG 1380
 441  G  L  A  R  M  E  L  F  L  L  L  S  A  I  L  Q  H  F  N  L 460

1381 AAGCCTCTCGTTGACCCAAAGGATATCGACATCAGTCCTGTAAATATTGGGTTTGGGTGT 1440
 461  K  P  L  V  D  P  K  D  I  D  I  S  P  V  N  I  G  F  G  C 480

1441 ATCCCACCACGTTTCAAACTCTGTGTCATTCCCCGTTCATGA 1482
 481  I  P  P  R  F  K  L  C  V  I  P  R  S  *  493
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Fig. 1. Nucleotide and deduced amino acid sequences of monCYP2E1. The sequences identified in this study are in bold.

comparison, INV*Sc*1 transformants with pYES2/CT as a null vector were used as a negative control. The cell lysates were centrifuged at 9000 *g* for 20 min at 4 °C, and the supernatants were further centrifuged at 105,000 *g* for 60 min at 4 °C. The resulting microsomal pellets were resuspended in 50 mM potassium phosphate buffer (pH 7.4) containing 20% glycerol and stored at −80 °C until use.

Assay for CYP2E1 holo- and apoproteins

Microsomal fractions were diluted to a protein concentration of 1.0 mg/ml (liver microsomes) or 5.0 mg/ml (yeast cell microsomes expressing CYP2E1s) with 100 mM potassium phosphate buffer (pH 7.4) containing 20% (v/v) glycerol and 0.4% (w/v) Emulgen 911, and total functional CYP contents 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 fractions (5.0 μg protein) 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 1:5000) as the secondary antibody. Immunoreactive proteins were visualized with chemifluorescence (enhanced chemiluminescence-plus reagents), and the band densities were relatively determined with Scion Image v4.0 (Scion Corporation, Frederick, MD).

Assay for CYP2E1-dependent enzymatic activities

Chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities were determined by high-performance liquid chromatography (HPLC) as described previously with some modifications (Lucas et al., 1993; Tassaneeyakul et al., 1993b). The incubation mixture contained chlorzoxazone (50–5000 μM) or 4-nitrophenol (50–5000 μM) as a substrate, liver microsomes (200 μg protein/ml) or 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. Each substrate was dissolved in methanol/dimethyl sulfoxide (50:50, v/v). The final concentration of the organic

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humCYP2E1   1 MSALGVTVALLVWAAFLLLVSMWRQVHSSWNLPPGPFPLPIIGNLFQLELKNIPKSFTRL 60
monCYP2E1   1 MSALGVSVALLVWVAVLLLVSIWRQVHSSWNLPPGPFPLPIIGNLFQLEWKNIPKSFTRL 60
              ****** ****** * ***** ************************** **********

                                                    SRS-1
humCYP2E1  61 AQRFGPVFTLYVGSQRMVVMHGYKAVKEALLDYKDEFSGRGDLPAFHAHRDRGIIFNNGP 120
monCYP2E1  61 AQRFGPVFTLYVGSRRVVVVHGYKAVREVLLDHKDEFSGRGDIPAFHAHRDRGIIFNNGP 120
              ************** * ** ****** * *** ********* *****************

humCYP2E1 121 TWKDIRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
monCYP2E1 121 TWKDTRRFSLTTLRNYGMGKQGNESRIQREAHFLLEALRKTQGQPFDPTFLIGCAPCNVI 180
              **** ********************************************************

                                  SRS-2                                 SRS-3
humCYP2E1 181 ADILFRKHFDYNDEKFLRLMYLFNENFHLLSTPWLQLYNNFPSFLHYLPGSHRKVIKNVA 240
monCYP2E1 181 ADILFRKHFDYNDEKFLRLMYLFNENFQLLSTPWLQLYNNFPSLLHYLPGSHRKVMKNVA 240
              *************************** *************** *********** ****

                                                                SRS-4
humCYP2E1 241 EVKEYVSERVKEHHQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
monCYP2E1 241 EIKEYVSERVKEHLQSLDPNCPRDLTDCLLVEMEKEKHSAERLYTMDGITVTVADLFFAG 300
              * *********** ********************************************

humCYP2E1 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
monCYP2E1 301 TETTSTTLRYGLLILMKYPEIEEKLHEEIDRVIGPSRIPAIKDRQEMPYMDAVVHEIQRF 360
              ************************************************************

              SRS-5
humCYP2E1 361 ITLVPSNLPHEATRDTIFRGYLIPKGTVVVPTLDSVLYDNQEFPDPEKFKPEHFLNENGK 420
monCYP2E1 361 ITLVPSNLPHEATRDTIFRGYIIPKGTVIVPTLDSVLYDNQEFPDPEKFKPEHFLDESGK 420
              ******************** ****** ************************* * **

                                                                SRS-6
humCYP2E1 421 FKYSDYFKPFSTGKRVCAGEGLARMELFLLLCAILQHFNLKPLVDPKDIDLSPIHIGFGC 480
monCYP2E1 421 FKYSDYFKPFSAGKRVCAGEGLARMELFLLLSAILQHFNLKPLVDPKDIDISPVNIGFGC 480
              *********** ****************** ****************** **  *****

humCYP2E1 481 IPPRYKLCVIPRS 493
monCYP2E1 481 IPPRFKLCVIPRS 493
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Fig. 2. Deduced amino acid sequence alignment of humCYP2E1 and monCYP2E1. The asterisks indicate the same amino acid residues between humCYP2E1 and monCYP2E1. SRSs are shown in shaded boxes.

solvent (methanol and/or dimethyl sulfoxide) in the incubation mixture was 1% (v/v). The reaction was initiated by the addition of microsomes from livers or yeast cells expressing CYP2E1 after preincubation at 37 °C for 1 min. After incubation at 37 °C for 20 min, the reaction was terminated by the addition of 4 ml of ethyl acetate. Samples for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities were spiked with internal standards (2 nmol of 4-nitrophenol and 5 nmol of salicylamide, respectively) and vigorously vortexed for 2 min. After centrifugation at 2000 *g* for 15 min, the organic phase was evaporated to dryness under a gentle stream of nitrogen at 35 °C. The residues were dissolved in 200 μl of methanol–water (50:50, v/v) and analyzed by HPLC.

The HPLC system consisted of an AS-8021 autosampler (Tosoh, Tokyo, Japan), a DP-8020 pump (Tosoh), a CO-8020 column oven (Tosoh), a UV-8020 UV detector (Tosoh) and a GT-102 degasser (Lab-Quatec, Tokyo, Japan) equipped with an Inertsil ODS-80A column (4.6 mm i.d. × 150 mm; GL Sciences, Tokyo, Japan). The column was maintained at 40 °C. Data acquisition was accomplished using LC-8020 v1.3 software (Tosoh). The product (6-hydroxychlorzoxazone) in the assay for chlorzoxazone 6-hydroxylation activity was eluted isocratically with 80 mM acetic acid–acetonitrile (82:18, v/v) at a flow rate of 1.0 ml/min. UV detection was performed at 295 nm. Retention times of 6-hydroxychlorzoxazone, chlorzoxazone and 4-nitrophenol were 5.6, 26.6 and 15.8 min, respectively. The limit of quantification for 6-hydroxychlorzoxazone was 10 pmol/ml. The 6-hydroxychlorzoxazone formation was linear for at least 30 min in microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys (200 μg/ml for liver microsomes and 500 μg/ml for yeast cell microsomes expressing CYP2E1s). In the assay for 4-nitrophenol 2-hydroxylation activity, the product (4-nitrocatechol) was eluted isocratically with 20 mM triethylamine–acetonitrile–methanol (82:12:6, v/v/v) at a flow rate of 1.0 ml/min. The pH of the aqueous portion was adjusted to 3.0 with phosphoric acid. UV absorption was monitored at 250 nm. Retention times of 4-nitrocatechol, 4-nitrophenol and salicylamide were 11.9, 22.6 and 9.1 min, respectively. The limit of quantification for 4-nitrocatechol was 20 pmol/ml. The 4-nitrocatechol formation was linear for at least 30 min in microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys (200 μg/ml for liver microsomes and 500 μg/ml for yeast cell microsomes expressing CYP2E1s). Standard curve samples for both assays were prepared in the same manner as the incubation samples. 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 livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys were determined by the method of Lowry et al. (1951) using bovine serum albumin as a standard. OR activities were measured as described previously, with a substrate concentration of 50 μM (Phillips and Langdon, 1962).

Data analysis

Kinetic parameters such as K_m and V_{max} for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation were estimated by analyzing Michaelis–Menten plots using Prism v4.0 software (GraphPad Software, San Diego, CA). Intrinsic clearance values were determined as the ratio of V_{max}/K_m. All values are expressed as the mean ± S.D. of three donors/animals or three separate experiments derived from independent preparations. Statistical comparisons were made with Student's *t*-test, and differences were considered statistically significant when the p value was <0.05.

Results

Sequence analysis

Sequence analysis determined that humCYP2E1 and monCYP2E1 cDNAs have an open reading frame of 1484 bp, and cDNAs encode the respective proteins of 493 amino acids. Fig. 1 shows the nucleotide and deduced amino acid sequences of monCYP2E1. As shown in Fig. 2, there are 28 different

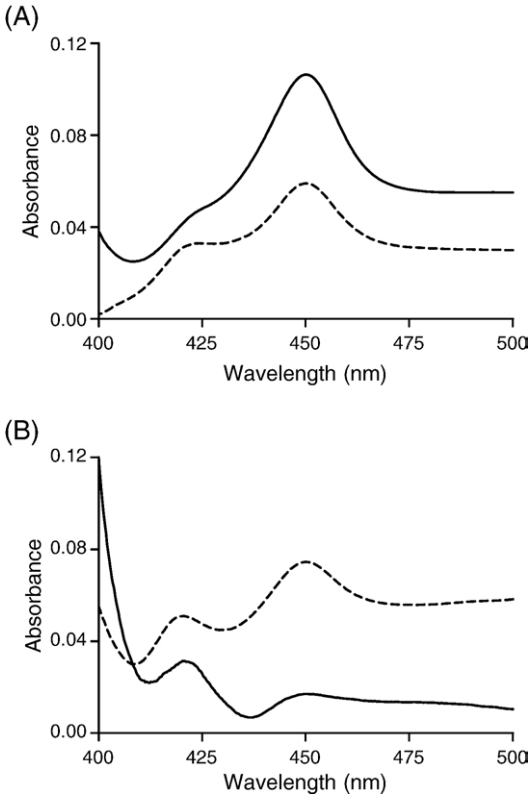

Fig. 3. Reduced CO-difference spectra of microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys. (A) Liver microsomes. (B) Yeast cell microsomes expressing CYP2E1s. Typical results of pooled microsomes from three independent preparations. The microsomal protein concentrations used were 1.0 mg/ml for liver microsomes and 5.0 mg/ml for yeast cell microsomes expressing CYP2E1s. Broken line, human liver microsomes or humCYP2E1; solid line, cynomolgus monkey liver microsomes or monCYP2E1.

Table 2
CYP contents and OR activities in microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys

Enzyme	CYP		OR [c]
	Reduced CO [a]	WB [b]	
HLM	316±128	100±19.7	209±48
MLM	563±105	74.1±11.0	145±22
humCYP2E1	38.4±19.4	100±13.4	233±98
monCYP2E1	16.4±2.7	69.4±16.0	288±88

Each value represents the mean±S.D. of three separate experiments derived from independent preparations. HLM, human liver microsomes; MLM, cynomolgus monkey liver microsomes.
[a] Reduced CO-difference spectral analysis (pmol/mg protein).
[b] Western blot analysis (% of HLM or humCYP2E1).
[c] nmol/min/mg protein.

amino acid residues between humCYP2E1 and monCYP2E1, with homology of 94.3% in the amino acid sequence.

Expression of humCYP2E1 and monCYP2E1 enzymes

CYP contents of microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys were examined by reduced CO-difference spectral and Western blot analyses. The reduced CO-difference spectra of yeast cell microsomes expressing humCYP2E1 and monCYP2E1 as well as human and cynomolgus monkey liver microsomes showed a Soret peak at around 450 nm (Fig. 3). CYP contents of enzyme sources in humans were 316 pmol/mg protein for liver microsomes and 38.4 pmol/mg protein for yeast cell microsomes expressing humCYP2E1. The levels in microsomes from livers and yeast cells expressing monCYP2E1 in cynomolgus monkeys were 1.78-fold and 42.7% of those in humans, respectively, although no significant differences were statistically observed (Table 2). The expression levels of CYP2E1 proteins in liver and yeast cell microsomes of humans and cynomolgus monkeys were also assessed by Western blot analysis which recognized both holo- and apoforms (Fig. 4). All enzyme sources except the negative control (mock) yielded immunodetectable CYP2E1 protein; however, the staining band intensities of microsomes from cynomolgus monkey livers and yeast cells expressing monCYP2E1 were weak compared with those of enzyme sources in humans. The relative levels (the summation of upper and lower bands) were human liver microsomes (100) and cynomolgus monkey liver microsomes (74.1), and humCYP2E1 (100) and monCYP2E1 (69.4), where human values were normalized to 100 (Table 2).

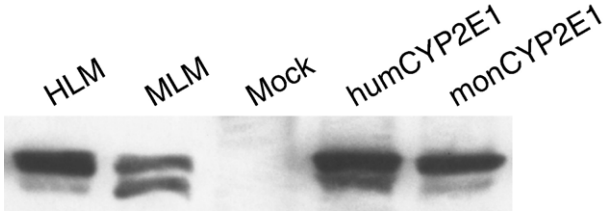

Fig. 4. Immunoblotting of microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys. Typical results of pooled microsomes from three independent preparations. The microsomal protein levels applied were 5.0 μg/lane for both microsomes from livers and yeast cells expressing CYP2E1s. HLM, human liver microsomes; MLM, cynomolgus monkey liver microsomes.

Table 3
Kinetic parameters for chlorzoxazone 6-hydroxylation by microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys

Enzyme	K_m [a]	V_{max}		V_{max}/K_m	
		Protein [b]	CYP [c]	Protein [d]	CYP [e]
HLM	822±322	909±130		1250±560	
MLM	418±42	532±56**		1270±50	
humCYP2E1	627±19	170±37	4.88±1.34	272±61	7.78±2.13
monCYP2E1	708±115	101±10*	6.29±1.67	143±14*	8.81±0.98

Each value represents the mean±S.D. of three separate experiments derived from independent preparations. HLM, human liver microsomes; MLM, cynomolgus monkey liver microsomes.
*$p<0.05$, **$p<0.01$ compared with HLM or humCYP2E1.
[a] μM.
[b] pmol/min/mg protein.
[c] pmol/min/pmol CYP.
[d] nl/min/mg protein.
[e] nl/min/pmol CYP.

OR activities in microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys were also determined. OR activities of enzyme sources in humans were 209 nmol/min/mg protein for liver microsomes and 233 nmol/min/mg protein for yeast cell microsomes expressing humCYP2E1. The activities in microsomes from livers and yeast cells expressing monCYP2E1 in cynomolgus monkeys were comparable to those in humans; there were no significant differences in the OR activities of humans and cynomolgus monkeys in any enzyme source (Table 2).

Enzymatic properties of humCYP2E1 and monCYP2E1

To obtain information on the enzymatic properties of human and cynomolgus monkey CYP2E1s, kinetic analyses for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation by yeast cell microsomes expressing humCYP2E1 and monCYP2E1 as

Table 4
Kinetic parameters for 4-nitrophenol 2-hydroxylation by microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys

Enzyme	K_m [a]	V_{max}		V_{max}/K_m	
		Protein [b]	CYP [c]	Protein [d]	CYP [e]
HLM	422±133	1250±570		2990±1090	
MLM	434±58	646±142		1480±210	
humCYP2E1	514±134	139±31	3.98±1.11	277±64	8.41±4.47
monCYP2E1	736±66	65.2±2.8*	4.04±0.71	88.8±6.0**	5.46±0.51

Each value represents the mean±S.D. of three separate experiments derived from independent preparations. HLM, human liver microsomes; MLM, cynomolgus monkey liver microsomes.
*$p<0.05$, **$p<0.01$ compared with HLM or humCYP2E1.
[a] μM.
[b] pmol/min/mg protein.
[c] pmol/min/pmol CYP.
[d] nl/min/mg protein.
[e] nl/min/pmol CYP.

well as by liver microsomes from humans and cynomolgus monkeys were performed. No activity in yeast cell microsomes of the negative control was detected with any substrate (data not shown). The calculated kinetic parameters estimated by Michaelis–Menten plots are summarized in Tables 3 and 4. The K_m values of human and cynomolgus monkey liver microsomes for chlorzoxazone 6-hydroxylation were 822 and 418 μM, respectively. The K_m values of yeast cell microsomes expressing humCYP2E1 and monCYP2E1 were 627 and 708 μM, respectively. There were no significant differences in the K_m values of liver and yeast cell microsomes between humans and cynomolgus monkeys. The V_{max} and V_{max}/K_m values of enzyme sources in humans on the basis of the microsomal protein level were 909 pmol/min/mg protein and 1250 nl/min/mg protein for liver microsomes, and 170 pmol/min/mg protein and 272 nl/min/mg protein for yeast cell microsomes expressing humCYP2E1, respectively. The V_{max} values of cynomolgus monkeys were significantly lower than those of humans (58.5–59.4%), although the V_{max}/K_m value of cynomolgus monkey liver microsomes was comparable to that of human liver microsomes. When the activities were normalized to CYP holoprotein levels to assess the intrinsic function of CYP2E1 enzymes, the V_{max} and V_{max}/K_m values of humCYP2E1 were 4.88 pmol/min/pmol CYP and 7.78 nl/min/pmol CYP, respectively. No significant differences in V_{max} and V_{max}/K_m values on the basis of CYP holoprotein level were observed between humCYP2E1 and monCYP2E1.

The K_m values of human and cynomolgus monkey liver microsomes for 4-nitrophenol 2-hydroxylation were 422 and 434 μM, respectively. The K_m values of yeast cell microsomes expressing CYP2E1s were 514 μM for humCYP2E1 and 736 μM for monCYP2E1, and there were no significant differences in the K_m values of humans and cynomolgus monkeys in any enzyme source. V_{max} and V_{max}/K_m values of enzyme sources in humans on the basis of microsomal protein level were 1250 pmol/min/mg protein and 2990 nl/min/mg protein for liver microsomes, and 139 pmol/min/mg protein and 277 nl/min/mg protein for yeast cell microsomes expressing humCYP2E1, respectively. The levels of cynomolgus monkeys were 32.1–51.7% of those in humans. V_{max} and V_{max}/K_m values of yeast cell microsomes expressing CYP2E1s on the basis of CYP holoprotein level were 3.98 pmol/min/pmol CYP and 8.41 nl/min/pmol CYP for humCYP2E1, and 4.04 pmol/min/pmol CYP and 5.46 nl/min/pmol CYP for humCYP2E1. V_{max} and V_{max}/K_m values of monCYP2E1 on the basis of CYP holoprotein level were comparable to those of humCYP2E1.

Discussion

CYP2E1, a member of the CYP superfamily, toxicologically plays a key role in the oxidative metabolism of various drugs, precarcinogens and solvents (Gonzalez, 1990; Guengerich et al., 1991). CYP2E1 activity and expression have also been shown to be influenced by genetic, physiological and environmental factors (Gonzalez, 1990; Kim and O'Shea, 1995; Lucas et al., 1995, 1998; McCarver et al., 1998). Although monkeys, including cynomolgus monkeys, are commonly used as an animal model for the development of medicines, and particularly for the characterization of pharmacokinetics and toxicological properties of novel molecules, little is known about the enzymatic properties of monkey CYP enzymes. The functional evaluation of human and cynomolgus monkey CYP2E1 enzymes should provide important information for the prediction and extrapolation of xenobiotic metabolism. Enzymatic activities in microsomes from yeast cells expressing human and cynomolgus monkey CYP2E1s were therefore investigated by kinetic analysis. To this end, we used chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation as probes for CYP2E1.

The expression of CYP2E1 protein was confirmed by Western blot analysis. Anti-human CYP2E1 antibody cross-reacted with two proteins in microsomes from livers and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys, and the staining intensities of both bands of cynomolgus monkeys were slightly weaker than those of humans. Multiple CYP2E1-like bands have been previously detected in ethanol-induced rat livers and phenobarbital-induced monkey brains (Neve and Ingelman-Sundberg, 1999; Lee et al., 2006). The profile of detected protein bands was also different between microsomes from livers and yeast cells expressing CYP2E1s. The results obtained in this study agreed with those of previous reports, and this phenomenon may be due the alteration of posttranslation modifications or cellular targeting. Furthermore, 450-nm absorbance was measured by reduced CO-difference spectra in yeast cell microsomes as well as in liver microsomes. The functional CYP level of monCYP2E1 was lower than that of humCYP2E1, although no significance was observed. Thus, the expression of functional CYP2E1 proteins in yeast cell microsomes expressing humCYP2E1 and monCYP2E1 as well as in human and cynomolgus monkey liver microsomes was confirmed.

Chlorzoxazone and 4-nitrophenol have been suggested to be typical substrates for CYP2E1 (Rendic and Di Carlo, 1997). Enzymatic activities in this study were analyzed in two ways: as pmol/min/mg protein (on the basis of microsomal protein level), and as pmol/min/pmol CYP (on the basis of CYP holoprotein level). Both yeast cell microsomes expressing humCYP2E1 and monCYP2E1 were capable of oxidizing chlorzoxazone and 4-nitrophenol as well as human and cynomolgus monkey liver microsomes at the substrate concentrations examined. We confirmed that the kinetics for these reactions in microsomes from liver and yeast cells expressing CYP2E1s of humans and cynomolgus monkeys fits the Michaelis–Menten equation.

K_m values for CYP2E1-dependent reactions of liver microsomes in previous studies have been estimated as follows: chlorzoxazone 6-hydroxylation, 39 μM for humans and 77 μM for cynomolgus monkeys; 4-nitrophenol 2-hydroxylation, 30 μM for humans and 14 μM for cynomolgus monkeys (Peter et al., 1991; Tassaneeyakul et al., 1993a; Amato et al., 1998). K_m values in this study (chlorzoxazone 6-hydroxylation, 820 μM for humans and 420 μM for cynomolgus monkeys; 4-nitrophenol 2-hydroxylation, 420 μM for humans and 430 μM for cynomolgus monkeys) were higher than those previously

reported in both species, although the cause of this difference is unclear at present. With respect to recombinant CYP2E1 enzymes, K_m values of human CYP2E1 enzyme expressed in heterologous cells such as *T. ni*, COS-1, COS-7 and human B-lymphoblastoid cells have been reported to be 81–660 μM for chlorzoxazone 6-hydroxylation and 21–141 μM for 4-nitrophenol 2-hydroxylation (Tassaneeyakul et al., 1993a; Fairbrother et al., 1998; Shimada et al., 1999; Hanioka et al., 2003). Thus, K_m values of human CYP2E1 enzyme for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation varied among heterologous cell expression systems, and K_m values of human and cynomolgus monkey CYP2E1 enzymes expressed in yeast cells were first estimated in this study. Additionally, there were no significant differences in K_m values of liver microsomes for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation between humans and cynomolgus monkeys. K_m values of yeast cell microsomes expressing humCYP2E1 and monCYP2E1 were comparable to those of human and cynomolgus monkey liver microsomes, respectively, suggesting that CYP2E1 is the predominant isoform responsible for oxidation in both species.

There were no notable differences in V_{max} and V_{max}/K_m values of liver microsomes for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation between humans and cynomolgus monkeys. In yeast cell microsomes expressing CYP2E1s, V_{max} and V_{max}/K_m values of monCYP2E1 on the basis of the microsomal protein level were significantly lower than those of humCYP2E1 in both reactions, whereas kinetic parameters on the basis of CYP holoprotein level exhibited similar values between humCYP2E1 and monCYP2E1. The low V_{max} values of monCYP2E1 on the basis of microsomal protein compared with those of humCYP2E1 are suggested to be attributed to differences in CYP2E1 holoprotein levels but not in the intrinsic enzyme function.

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 for human CYP2E1 has been generated using the CYP2C5 crystal structure 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). The distance between oxidation sites on substrate molecules (6-position for chlorzoxazone; 2-position for 4-nitrophenol) and haem iron was 5.7 Å. Phe205 and Phe298 of CYP2E1 are able to form π–π stacking with the benzoxazone ring of chlorzoxazone or benzene ring of 4-nitrophenol. Asp202 and Thr303 of CYP2E1 in chlorzoxazone 6-hydroxylation enter into hydrogen bonding with hydroxyl and chloro groups of the substrate, respectively. With respect to 4-nitrophenol 2-hydroxylation, Asp202 and Thr303 residues serve to anchor the 4-nitrophenol molecule via nitro and hydroxyl groups, respectively. These amino acid residues were in SRS-2 or SRS-4, and were identical between humCYP2E1 and monCYP2E1. Accordingly, it is considered that the functional similarities of humCYP2E1 and monCYP2E1 are due to the identity of amino acid resides interacting with substrates. Further studies are required to elucidate the relationship between the protein structure and substrate specificity of primate CYP2E1s.

In conclusion, we expressed human and cynomolgus monkey CYP2E1 enzymes in yeast cells, and showed chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation activities in yeast cell microsomes expressing CYP2E1s as well as in liver microsomes of humans and cynomolgus monkeys. The functional CYP levels of monCYP2E1 expressed in yeast cell microsomes were lower than those of humCYP2E1 in both reduced CO-difference spectral and Western blot analyses, although no significant differences were statistically observed. There were no significant differences in K_m values for chlorzoxazone 6-hydroxylation and 4-nitrophenol 2-hydroxylation between humans and cynomolgus monkeys in both microsomes from livers and yeast cells expressing CYP2E1s. V_{max} and V_{max}/K_m values of monCYP2E1 on the basis of microsomal protein level were significantly lower than those of humCYP2E1 in both reactions, whereas the kinetic parameters of humCYP2E1 and monCYP2E1 on the basis of CYP holoprotein level exhibited similar values. These findings suggest that human and cynomolgus monkey CYP2E1 enzymes have high homology in their amino acid sequences, and that their enzymatic properties are considerably similar. The information gained in this study should help with in vivo extrapolation and to assess the toxicity of xenobiotics.

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