MINIPIG CYTOCHROME P450 2E1: COMPARISON WITH HUMAN ENZYME

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ABSTRACT:

Cytochrome P450 2E1 (CYP2E1) is an enzyme of the CYP2 family of cytochrome P450 heme enzymes known to be responsible for metabolic activation of many low molecular weight compounds suspected to act as chemical carcinogens. It is a major catalyst of the oxidation of benzene, styrene, carbon tetrachloride, chloroform, dichloromethane and other chlor derivatives of methane and ethane, and of vinyl chloride, and is known to take part in metabolic activation of nitrosamines (Guengerich et al., 1991). CYP2E1 metabolizes volatile anesthetics (halothane, enflurane) and some drugs of relatively small molecules such as acetaminophen and isoniazid (Lieber, 1997). Its physiological role is apparently connected with regulation of energy balance and of the lipid metabolism, since it is induced by starvation and diabetes (Lieber, 1997). CYP2E1 is known to metabolize ethanol; it is also inductable by ethanol and by some endogenous compounds such as acetone. CYP2E1 is expressed in human fetus early in development, which indicates its importance for the developing human organism (Johnsrud et al., 2003). It is one of the major hepatic P450 enzymes; however, it has also been detected in significant levels in human esophagus, kidney, lung (Lieber, 1997), and brain (Upadhya et al., 2000).

Pig and minipig liver microsomal P450 enzymes have been studied in more detail recently. P450 enzymes were shown to be involved in metabolism of veterinary drugs (Monshouwer et al., 1998). Moreover, their properties are intensively studied since P450 activities belong to important functional characteristics of minipig or pig livers (Anzenbacher et al., 1998; Skaanild and Friis, 1999; Soucek et al., 2001) and may be compared with the respective metabolites. The experiments performed with both the liver microsomal fraction and reconstituted systems with human or minipig CYP2E1 confirmed the similarity of both enzymes. Inhibition with diethyldithiocarbamate gave comparable K_i values for minipig as well as for the human CYP2E1. The results indicate that the systems containing minipig CYP2E1 may be used to model the respective CYP2E1-catalyzed reactions of drug metabolism in humans.

Myers et al. (2001) have investigated the properties of the market weight swine P450 enzymes in liver microsomes and in the S10 fraction by Western blot; also, enzyme activities specific for individual P450 enzymes have been determined in subcellular preparations. Interest in pig or minipig hepatocytes and P450s increased over the last few years since they seem to be suitable models in experimental pharmacology (Václavíková et al., 2004) and, moreover, pig hepatocytes are possibly intended also for xenotherapy or for construction of bioartificial liver supporting devices (Desille et al., 1999; Donato et al., 1999; Couzin, 2002).

To date, only one successful attempt to obtain partially purified minipig liver microsomal drug-metabolizing P450 enzymes has been published. Soucek et al. (2001) confirmed that the minipig CYP3A (assigned as CYP3A29; Protein Data Bank Accession Number AF424780) effectively converts prototypic human CYP3A4 substrate nifedipine to the respective metabolite and that the minipig CYP2A enzyme metabolizes coumarin, a characteristic substrate of the human CYP2A6. To get more detailed information on individual P450 enzymes from pig or minipig, purified CYP2E1 has been isolated from minipig liver microsomes for the first time, and its structural and enzymological characteristics are described and compared with properties of the human orthologous form.

Materials and Methods

Chemicals. All reagents and chemicals were reagent grade and were obtained from Sigma-Aldrich (Prague, Czech Republic) if not stated otherwise. The chemiluminiscence kit for Western blotting (Immun Star) was purchased from Amersham Biosciences UK, Ltd. (Little Chalfont, Buckinghamshire, UK). Goat anti-human CYP2E1 antibody was obtained from Daichi Pure Chemicals (Tokyo, Japan). BCA protein determination kit was obtained from Pierce (Rockford, IL). Human NADPH/cytochrome P450 oxidoreductase (P450 reductase) was purchased from Sigma-Aldrich; minipig enzyme was prepared from fractions obtained during the first step of minipig P450 isolation by the method of Soucek et al. (2001). P450 reductase was then isolated by affinity chromatography (Yasukochi and Masters, 1976).

ABBREVIATIONS: P450, cytochrome P450; PCR, polymerase chain reaction.
Western blotting using the rat anti-CYP2E1 IgG. Selected content typically over 12 nmol P450/mg protein. The course of purification has been followed by electrophoresis (Mini Protean apparatus; Bio-Rad) and typically over 12 nmol P450/mg protein. The course of purification has been followed by electrophoresis (Mini Protean apparatus; Bio-Rad) and Western blotting using the rat anti-CYP2E1 IgG.

**Purification of Minipig CYP2E1.** Minipig microsomes were obtained from untreated minipig livers (Brno white variety of Goettingen minipigs; Research Institute of Veterinary Medicine, Brno, Czech Republic; 25–30 kg body weight, male castrates, n = 5, age 6 months; approved by local and national ethical commissions) by a standard procedure (Lake, 1990). Microsomal fraction was solubilized by cholate and applied onto the octylamino Sepharose CL-4B column. The isolation method generally followed the one described earlier (Soucek et al., 2001). The last peak eluted from the octylamino Sepharose column (eluted with buffer (pH 7.25) containing 0.1 M K-phosphate, 0.05 mM EDTA, 20% (v/v) glycerol. Elution of CYP2E1 was achieved by a linear gradient of K-phosphate (10 mM to 300 mM) in the equilibration buffer with 0.3% Triton N-101. As a rule, this procedure had to be repeated twice to get electrophoretically pure CYP2E1 protein with specific content typically over 12 nmol P450/mg protein. The course of purification has been followed by electrophoresis (Mini Protein apparatus; Bio-Rad) and Western blotting using the rat anti-CYP2E1 IgG.

**Minipig CYP2E1 cDNA Cloning and Sequencing.** Total RNA from minipig liver was isolated by TRIzol according to the procedure supplied by the producer (Invitrogen, Paisley, UK). RNA quality and quantity were assessed by UV-visible spectrophotometry and agarose gel electrophoresis. cDNA was then synthesized using 1 μg of total RNA with the help of a kit purchased from MBI Fermentas ( Vilnius, Lithuania). Minipig cDNA was amplified by PCR with forward, 5’-atcgatcagagggcttcagttgtaagttggctgg-3’ and reverse, 5’-gtggcctttcgtcttcaaatgatcagtagttgtaagttggctgg-3’ primers designed using the known pig CYP2E1 sequence (GenBank Accession Number AB052259). The first 11 codons of forward primer were changed (the only primary structure change created was the second amino acid to Ala) to prevent unfavorable secondary structure formation during subsequent protein expression trials, and the reverse primer contained a 6-His tag sequence to prevent unfavorable secondary structure formation during subsequent protein expression trials, and the reverse primer contained a 6-His tag sequence for direct cloning and expression in Escherichia coli (Invitrogen). Forward primer also contained the NdeI restriction site and reverse primer, the Sall restriction site (both positions underlined) for subcloning into expression vector pC'W previously used for expression of human P450s (Sandhu et al., 1993). Conditions of PCR cycling were 3 min of denaturation at 94°C, 10 cycles of 30 s at 94°C, touchdown annealing for 30 s at 60°C, gradually decreasing to 50°C, 2 min at 72°C followed by 25 cycles of 30 s at 94°C, 30 s at 65°C, 2 min at 72°C with 1.5 mM MgCl₂ in the general reaction mixture. The resulting PCR product was further amplified in nested PCR containing forward, 5’-GGGGACAAGTTTGTACAAAAAAGC-AGGGTACatcgatcagagggcttcagttgtaagttggctgg-3’ and reverse, 5’-GGGGACACCTTGTGACAAAAGCTTGatcgatcagagggcttcagttgtaagttggctgg-3’ primers. Both primers carried a sequence compatible with the specific recombination sites of the Gateway system (sequences in capital letters) for direct cloning and expression in Escherichia coli (Invitrogen). Forward primer also contained the NdeI restriction site and reverse primer, the Sall restriction site (both positions underlined) for subcloning into expression vector pC’W previously used for expression of human P450s (Sandhu et al., 1993). Conditions of PCR cycling were 3 min of denaturation at 94°C, 10 cycles of 30 s at 94°C, touchdown annealing for 30 s at 60°C, gradually decreasing to 50°C, 2 min at 72°C followed by 25 cycles of 30 s at 94°C, 30 s at 72°C with 2.5 mM MgCl₂ in the general reaction mix. For both PCRs, Platinum Pfx polymerase with proofreading activity was used (Invitrogen). Minipig CYP2E1 coding sequence was then cloned into pDONR201 plasmid of the Gateway system (sequences in capital letters) for direct cloning and expression in E. coli (Invitrogen). The plasmid designated PigCYP2Eseq1 was transfected into E. coli JM109 cells for sequencing PCR were as follows: 5’-atcgatcagagggcagttgtaagttggctgg-3’ (pCWF), 5’-ggggacaagtttgtacaaaaaagc-aggccctttcgtcttcaaatgatcagtagttgtaagttggctgg-3’ (PigCYP2Eseq2), 5’-ctcgccaggtggtcaggttgc-3’ (PigCYP2Eseq3), 5’-ttgattcagagggcttcagttgtaagttggctgg-3’ (PigCYP2Eseq4), 5’-agggccctttcgtcttcaaatgatcagtagttgtaagttggctgg-3’ (pC’W). Sequence analysis was carried out on ABI Prism 310 Genetic Analyzer (Applied Biosystems) equipped with 310 Data Collection Software, DNA Sequencing Analysis software (both Applied Biosystems), and the Lasergene program for processing of DNA sequence data of DNASTAR (Madison, WI). The sequence was then analyzed by the BLAST program (available at ncbi.nlm.nih.gov/blast) and compared with human and pig CYP2E1 sequences retrieved from GenBank, where it was submitted as minipig CYP2E1 with Accession Number AY581116.

**Enzyme Assays.** Chlorzoxazone 6-hydroxylating activity of CYP2E1 preparations in microsomes as well as in the reconstituted systems was determined according to the method of Lucas et al. (1996). Hydroxylation of p-nitrophenol was assessed by the method of Tassaneeyakul et al. (1993). For both methods, an HPLC system of Shimadzu (Tokyo, Japan) Class VP was used. The experiments were routinely performed in quadruplicate. Reconstitution systems were prepared as described by Shimada and Yamazaki (1998) using the respective reductases. Inhibition of chlorzoxazone 6-hydroxylation by diethylthiocarbamate was studied with microsomal and reconstitution systems, and the Kᵢ values were determined as averages from Dixon plots with three substrate concentrations used (corresponding to 0.5 Kᵢ, Kᵢ, and 2Kᵢ). Parameters of enzyme kinetics were obtained using LSW Data Analysis software (www.mdl.com) and Sigma Plot 8.0.2 (SPSS Inc., Chicago, IL).

**Results**

**CYP2E1 Structure.** Minipig CYP2E1 has been purified from liver microsomes to homogeneity (Fig. 1). The protein has been clearly recognized by commercial goat antibodies against human CYP2E1 by Western blot.

To confirm the identity of this enzyme, primary structure has been determined. Since the preliminary N-terminal sequencing experiments have confirmed the identity of the published pig (GenBank Accession No AB000885) and minipig CYP2E1 N-terminal sequences, it was possible to continue in obtaining the cDNA clone using the primers derived from the data for the pig enzyme. Sequencing of the cDNA has yielded a result published in GenBank (Accession Number AYS81116).

The primary structures of minipig and pig CYP2E1 are identical with one exception: in position 346, there is an aspartic acid residue in the minipig enzyme contrary to valine in the pig protein. Interestingly, this minor change makes the minipig enzyme more similar to the human protein because the corresponding amino acid residue in the human protein is the glutamic acid. The pig or minipig protein is

**FIG. 1.** Electrophoresis of purified proteins (Coomassie Blue stain). Lanes from left to right: 1, protein standards (mol. wt. 113, 92, 53, 35, and 21); 2, empty lane; 3, minipig CYP2E1; 4, minipig NADPH/cytochrome P450 oxidoreductase; 5 and 6, human CYP2E1.
composed of 496 amino acid residues; the human enzyme is two residues shorter. The minipig (or pig) CYP2E1 exhibits 79.6% primary sequence identity with the human counterpart. The most remarkable difference in the primary structure of (min)pig and human CYP2E1 is located close to the substrate recognition site region I (Gotoh, 1992). Here, two basic amino acids (His 92 and Asn 95) are present in minipig CYP2E1 instead of two aspartic acid residues. Similarly, at the beginning of the F helix (and of the substrate recognition site 2), there is a basic lysine residue in the minipig and pig enzyme (Lys 194), whereas a glutamic acid residue is present in the human CYP2E1. The differences in the primary structure of the (min)pig enzyme do not include the key amino acid residues predicted to interact with a prototypic substrate, chlorozoxazone, in the substrate binding site of human CYP2E1, according to Park and Harris (2003). For p-nitrophenol (another CYP2E1 substrate) bound in the active site of a human CYP2E1 model (Park and Harris, 2003), the respective amino acid changes are merely conservative (V364L, P213G, G479A). Also, the model of the CYP2E1 active site constructed on the basis of CYP2C5 (Lewis et al., 2003) exhibits the same amino acid residues in the active site in the human and minipig CYP2E1 enzyme.

**Enzyme Activities.** The relatively high degree of homology between the primary structure of human CYP2E1 and orthologous enzymes from other mammalian species is reflected in the similarity, also, in substrate specificity, although some interspecies differences exist (e.g., in metabolism of butadiene) (Lewis et al., 1997). We have investigated in detail the enzyme kinetics of chlorozoxazone 6-hydroxylation and of p-nitrophenol hydroxylation in minipig liver microsomal fraction as well as in the reconstituted system with CYP2E1 and P450 reductase from minipig. Human liver microsomal samples and the reconstituted system with human P450E1 and P450 reductase were used for comparison. Inhibition of CYP2E1 activities by diethyldithiocarbamate, a typical inhibitor of CYP2E1 of human origin as well as of other species (Court et al., 1997), was also investigated.

Results obtained with both prototypic substrates are displayed in the tables. In Table 1, data for enzyme kinetics of chlorozoxazone hydroxylation in liver microsomal fraction and in reconstituted systems of both species (human and minipig) are shown. Data obtained with another substrate, p-nitrophenol, are displayed in Table 2. The $K_M$ values for both substrates in the microsomal fraction and in the reconstituted system indicate lower affinity of the minipig enzyme; this is apparently true, also, when the $V_{max}/K_M$ ratio (often taken as a measure of enzyme specificity) is considered. With the exception of the $V_{max}$ for p-nitrophenol hydroxylation in the system reconstituted with minipig CYP2E1, data for minipig CYP2E1 show the differences less than 1 order of magnitude (approximately 4 times higher $K_M$ and 4 times lower $V_{max}$) when compared with those for the respective human counterparts. The differences observed may be ascribed to changes in the polarity (or even reversals in the polarity) of amino acid residues in the minipig enzyme observed in the vicinity of the “substrate recognition sites” (see the preceding paragraph). However, the data conclusively document the ability of minipig enzyme to metabolize chlorozoxazone as well as $p$-nitrophenol at rates comparable to the human CYP2E1.

Inhibition of CYP2E1 activities by diethyldithiocarbamate, although not specifically specific to this enzyme in humans as well as in other species (Bogaards et al., 2000; Sai et al., 2000), is another commonly used probe of similarity of the human and minipig enzyme. In this work, diethyldithiocarbamate has been shown to inhibit chlorozoxazone 6-hydroxylation in both human and minipig microsomes (with $K_i$ values of 21 and 38 μM, respectively) and reconstituted systems ($K_i$ values of 7 μM for human CYP2E1 and 20 μM for the minipig enzyme). Although the $K_i$ values of the respective human and minipig systems do not differ more than 3 times, the results for the reconstituted systems with only CYP2E1 present may indicate lower specificity of minipig CYP2E1 for this inhibitor.

**Discussion**

The results presented were obtained both with microsomal fraction and with the reconstituted systems, thus answering the principal question raised by Myers et al. (2001), whether the pig or minipig CYP2E1 is able to convert the prototypical human CYP2E1 substrates to the respective metabolites. Interestingly, specificity of the minipig enzyme corresponds to the properties of the human form: in humans, chlorozoxazone may also be converted (however, less effectively) to its 6-hydroxy metabolite by CYP1A1 (Carriere et al., 1993) as well as by CYP3A enzymes (Gorski et al., 1997). In line with this fact, there is in the minipig probably a contribution also from the CYP3A (Madden et al., 1998) as well as the CYP1A enzymes to the metabolism of chlorozoxazone (Bogaards et al., 2000) in the microsomal preparations. The second substrate, p-nitrophenol, is known to be metabolized by CYP2E1 as well as (with lower turnover number) by CYP3A enzymes in humans (Zerrilli et al., 1997). Most recent results indicate an involvement of CYP2A6 and possibly also of CYP2C19 enzymes in this reaction (Monostory et al., 2004). However, this activity is still recognized as the standard one for a CYP2E1 enzyme (Chang et al., 1998); hence, it should be tested with any new CYP2E1 species. The results presented here confirm the role of minipig CYP2E1 in this reaction (Table 2).

**Comparison of CYP2E1 of Minipig and of Other Species.** CYP2E1 is one of the most conserved P450 enzymes according to the primary structure, exhibiting nearly 80% sequence identity across species (Guentherich, 1997). High sequence similarity corresponds to similar catalytic properties of CYP2E1 enzymes of various origins (Chauret et al., 1997; Court et al., 1997; Bogaards et al., 2000; Zuber et al., 2002). Comparison of catalytic rates is, however, difficult, since the enzyme velocity is often expressed relative to protein instead of to the more specific P450 content. Literature data seem to indicate a species-conserved mechanism for oxidative biotransformation by CYP2E1 (Court et al., 1997). In this respect, minipig is apparently not an exception. In our hands, the $K_M$ values for 6-hydroxylation of chlorozoxazone in liver microsomal fraction ranged from 40 μM

### Table 1

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<thead>
<tr>
<th>Substrate</th>
<th>Microsomes</th>
<th>Reconstituted Systems</th>
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<tbody>
<tr>
<td>$K_M$ (μM)</td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td>$V_{max}$ (nmol · min⁻¹ · mmol P450⁻¹)</td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td>Chlorozoxazone</td>
<td>189 ± 20</td>
<td>622 ± 10</td>
</tr>
<tr>
<td>P-Nitrophenol</td>
<td>2.0 ± 0.3</td>
<td>4.0 ± 0.5</td>
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### Table 2

<table>
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<th>Substrate</th>
<th>Microsomes</th>
<th>Reconstituted System</th>
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<tbody>
<tr>
<td>$K_M$ (μM)</td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td>$V_{max}$ (nmol · min⁻¹)</td>
<td>Minipig</td>
<td>Human</td>
</tr>
<tr>
<td>Chlorozoxazone</td>
<td>121 ± 16</td>
<td>468 ± 17</td>
</tr>
<tr>
<td>P-Nitrophenol</td>
<td>1.1 ± 0.5</td>
<td>148.9 ± 19.8</td>
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(human) to 201 µM (pig), and for p-nitrophenol hydroxylation from 30 µM (dog) to 121 µM (minipig) (Anzenbacherova et al., 2005). However, molecular mechanisms of induction of CYP2E1 in various species may differ; e.g., β-naphthoflavone, a prototypical inducer of CYP1A, also has been shown to induce the CYP2E1 form in the dog (Jayosi et al., 1996). On the other hand, the CYP2E1 enzyme activities seem to be comparable in most of the species used in experimental pharmacology and toxicology, hence allowing for successful modeling of drug metabolism by CYP2E1 in humans.

References


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