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Purification, molecular cloning, heterologous expression and characterization of pig CYP1A2

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Abstract

Porcine cytochrome P450 (CYP) 1A2 was purified to electrophoretic homogeneity from the hepatic microsomes of β-naphthoflavone-treated male pigs. In a reconstituted system, this enzyme showed a good catalytic activity towards caffeine, acetanilide, and methoxyresorufin, all known markers of mammalian CYP1A2. Using 3′- and 5′-rapid amplification of coding DNA (cDNA) ends (RACE), we amplified from the liver RNA of control pigs a full-length 1827 bp cDNA containing an open reading frame of 1548 bp which encoded a putative CYP1A2 protein of 516 amino acids and an estimated Mr of 58 380 Da. Reverse transcriptase-polymerase chain reaction (RT-PCR) experiments showed that the messenger RNA (mRNA) of CYP1A2 was expressed in liver, heart and nasal mucosa but not in lung, small intestine, kidney and brain. Using the pCW vector containing a *N*-terminal modified cDNA, pig CYP1A2 was expressed in *Escherichia coli*. 3-[(3-Chloroamidopropyl)dimethylmmonio]-1-propane-sulfonate (CHAPS)-solubilized *E. coli* preparations expressing CYP1A2 produced a functionally isoform which, in a reconstituted system, was catalytically active toward ethoxyresorufin and methoxyresorufin showing K_m's similar to those obtained with CYP1A2 purified from pig liver or human recombinant CYP1A2. Taken together, these results demonstrate that domestic pigs have a functionally active CYP1A2 gene well expressed in the liver with biochemical properties quite similar to those corresponding to the human enzyme.

Keywords: *CYP1A2, pig, purification of CYP1A2*

Introduction

Cytochrome P450 (CYP or P450) comprises a superfamily of enzymes that play a decisive role in the oxidation of a great number of xenobiotics and endogenous substrates, and multiple forms of CYPs are present in mammals (Nelson et al. 1996).

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Among various CYPs, CYP1A2 has been of considerable interest because of its role in the metabolism of drugs (that is, caffeine, theophylline, phenacetin, and warfarin) and activation of aromatic and heterocyclic amines to reactive metabolites which have carcinogenic potential (Lewis and Lake 1996; Guengerich 1997). CYP1A2 is primarily a hepatic enzyme and it is known as one of the major CYP enzymes in the human liver where it represents about 10–13% of the total CYP content (Rendic and Di Carlo 1997). This enzyme is induced in rodents, and presumably in human, by polycyclic aromatic compounds, cigarette smoke, and heterocyclic amines formed from the pyrolysis of cooked meats (Buther et al. 1989).

CYP1A2 has been cloned, expressed or purified and characterized in various mammals including rat, mouse, cat, rabbit, monkey, and human (Guengerich 1997; Sakuma et al. 1997; Tanaka et al. 2006), but not in pig. Indeed, previous studies (Marini et al. 1998; Skaanild and Friis 1999; Myers et al. 2001) have shown in pig livers the constitutive expression of a single protein band recognized by antibodies raised against rat CYP1A1 or recombinant human CYP1A2, probably corresponding to CYP1A2. Thomsen et al. (1991) also described a partial purification of two CYP proteins of similar molecular weight (about 58 kDa) from β-naphthoflavone (βNF)-treated pig livers which are probably CYP1A1 and CYP1A2, but their catalytic activities have not been reported.

Recently, pig has attracted considerably attention as it is believed to be the best animal species in order to supply an external liver to the construction of bioartificial liver devices for patients waiting liver transplantation (Chari et al. 1994; Nelson et al. 1996). The use of pig is also increasing as an animal model to predict the kinetics and toxicity properties of drugs because of its similarity to human anatomy and physiology (Swindle and Smith 1998).

Various studies have shown that pig liver expresses the main drug-metabolizing enzyme activities similar to those of human (Anzenbacher et al. 1998; Donato et al. 1999; Soucek et al. 2001). However, detailed investigations on the enzymology of porcine CYP enzymes, necessary to extrapolate to humans the results found in pigs, are limited to a few isoforms (Lundell 2002; Lin et al. 2004; Sakuma et al. 2004; Baranova et al. 2005; Skaanild 2006). Therefore, it appeared important to study porcine CYP1A2 in detail to extend one's understanding of the drug-metabolizing capacity of the pig as a possible animal model in replacement of dog or monkey, species widely used in pharmacology and toxicology.

In the present study we report the purification of this enzyme from livers of βNF-treated male domestic pigs, the molecular cloning of cDNA encoding this porcine enzyme, its expression in *Escherichia coli*, and a detailed study of its enzymatic properties.

Materials and methods

Chemicals

Caffeine, 1,7-dimethylxanthine, beta-naphthoflavone, acetanilide, 2-aminofluorene, *n*-octylamine, 4-hydroxy-acetanilide, methoxyresorufin, resorufin, ethoxyresorufin, sodium cholate, 3-[(3-cholamidopropyl)-dimethylamonio]-1-propanesulfonate (CHAPS), and 3-morpholinopropanesulfonic acid (MOPS) were supplied from Sigma Chemical Co. (St Louis, MO, USA). Sepharose 4B and DEAE Sephacel were purchased from Pharmacia (Uppsala, Sweden), whereas DEAE cellulose (DE53) and carboxymethyl cellulose 52 (CM) were from Whatman (Clifton, USA) and hydroxyapatite (HA) was from Clarkson Co. (Williamsport, USA). Emulgen 911 was from CAO Corp. (Tokyo, Japan). Mouse monoclonal antibodies against recombinant human CYP1A2 were purchased from Panvera (Madison, WI, USA), whereas polycloned antibodies against rat CYP1A1 were

from Gentest (Woburn, MA, USA). All chemicals and reagents were of analytical grade. Tripure Isolation reagent and restriction enzymes were from Roche Molecular Biochemicals (Indianapolis, USA). ThermoScript III RT, Gene Racer Kit, Platinum Pfx *Taq* Polymerase were from Invitrogen Life Technologies (Carlsbad, CA, USA). Master Mix PCR, pGEM-T easy vector, Wizard® Plus SV MiniPrepsDNA Purification System were purchased from Promega (Madison, MI, USA). Peroxidase-conjugated anti-mouse immunoglobulin G (IgG) and oligonucleotides were obtained from Sigma-Aldrich (Milan, Italy). PCW Ori plus plasmid containing human CYP1A2 was kindly supplied by Professor F. P. Guengerich (Nashville, TN, USA). β-Nicotinamide adenine dinucleotide phosphate (NADPH)-cytochrome P450 reductase was purified from rats as previously reported (Amato et al. 1996).

Animal treatment and preparation of microsomes

This study was performed using male castrated Largewhite per Landrace hybrid domestic pigs (30–35 kg body weight) of about 3 months old. The animals, supplied by a commercial farm, were housed in floored indoor pens, received food and drinking water *ad libitum*, and were maintained on a 12-h light/dark cycle. After a preliminary period of 15–20 days to allow adjustment of the pigs to diet, temperature and humidity conditions, two pigs were given an intraperitoneal suspension of β-naphthoflavone (βNF) in corn oil ($30\,mg\,kg^{-1}$) for 4 days and were sacrificed 24 h after the final injection. Eight control pigs were given an intraperitoneal injection of the vehicle (corn oil) alone. Throughout the study all animals were under clinical observation. Liver microsomes were prepared as previously published (Longo et al. 1991) from two control and two βNF-treated animals. Microsomal protein concentration was measured by the method of Lowry et al. (1951) using bovine serum albumin as the standard. Surgical procedures and experimental protocols were approved by the Animal Care Committee of Bologna University.

Isolation of total RNA and cDNA synthesis

Total RNA was extracted from control pigs liver, lung, small intestine, heart, kidney, brain and nasal mucosa (80–100 mg of tissue) with TriPure Isolation Reagent, as recommended by the manufacturer. The resulting pellet was resuspended into 25–50 µl sterile DEPC water, as required. RNA (1 µg) was reverse transcribed, after DNAse treatment, with 1 U of ThermoScript RT in the presence of random hexanucleotide primers according to the manufacturer's instructions.

PCR and sequence analysis

The primers used to identify CYP1A2 and glyceraldehyde 6-phosphate dehydrogenase (GAPDH) were constructed by the OLIGO 4.0 program on the basis of conserved regions of homologous murine, rat and human genes retrieved from GeneBank; the CLUSTAL X program was used for multiple sequence alignment. A total of 2 µl of cDNA were added to a PCR Master Mix for the amplification reaction (35 cycles). The DNA fragments were separated on 1% agarose gel and subsequently stained with ethidium bromide. Polymerase chain reaction (PCR) products were purified by a Wizard SV Gel and PCR Clean-Up system, as indicated by the manufacturer, and were sequenced by automated fluorescent cycle sequencing by BMR Genomics Sequencing Core Service (University of Padua).

The sequence homology between pig and human genes was calculated by using the BLAST program.

CYP1A2 cDNA cloning and sequencing

Total RNA was prepared from liver of untreated pig with Tripure Isolation reagent and modified by a Gene Racer Kit and reverse transcribed with 1 U of ThermoScript III RT according to the manufacturer's instructions. Firstly, a CYP1A2 cDNA central fragment of 520 bp was cloned with two primers obtained by a multi-alignment strategy from the sequences encoding CYP1A2 isoforms of other species (forward primer 5′-GCCTGACCT CTACACCTC-3′ and reverse primer 5′-GTGATGTCCCGGACACTGTTC-3′). The PCR programme consisted of 35 cycles (20 s at 94°C, 30 s at 52°C and 1 min at 72°C) with Master Mix PCR. The full-length cDNA was obtained employing for the isolation of the missing 5′-end, the following two reverse primers: 5′-TCCCGATAGCGCTCCTGG ACCATT-3′ (racer primer) and 5′-TGGGCAGATATCTAAGGATGGGG-3′ (nested primer); and for the isolation of the missing 3′-end, the following two forward primers: 5′-AACACCTTCTCCATTGCCTCAGACC-3′ (racer primer) and 5′-TGATGGCAGGG CCTGGGCACTTT-3′ (nested primer). These primers were chosen from the central fragment sequence firstly isolated using proof-reading Platinum Pfx *Taq* Polymerase according to the Invitrogen Gene Racer Kit. The PCR products were gel purified and cloned into pGEM-T easy vector and sequenced. The homologies were performed with the NCBI-BLAST database.

Construction of expression plasmid for pig CYP1A2

For the expression of pig CYP1A2 cDNA, the nucleotide sequence encoding its wild-type *N*-terminal region was changed to include the MALLLV amino acids sequence and the *NdeI* site, as previously reported (Fisher et al. 1992), using the sense primer 5′-GCCCATATGGCTCGTTTATTAGCAGTTTTTTTCTCAGCCACAGAG-3′ and the antisense primer 5′-CTGGTAACTTCATTTGATGG-3′, containing the STOP codon. The PCR product was subcloned in pGEMT-easy vector for sequencing and to confirm the modified sequence. Finally, recombinant CYP1A2 cDNA was digested using *NdeI* site and *SalI* restriction site present in pGEMT-easy vector and ligated in the pCWOri^{+} vector.

Expression of pig CYP1A2 and human CYP1A2 pCWori^{+} plasmids

Plasmids were transformed in *Escherichia coli* DH5α cells. A single resistant colony of DH5αcells transformed with each plasmid was grown overnight a 37°C in Luria–Bertani (LB) medium containing 100 μg ml^{-1} of ampicillin. A 10 ml aliquot of LB pre-culture was inoculated into 1 litre of terrific broth (TB). The TB medium was supplemented with ampicillin (100 μg ml^{-1}), 1 mM thiamine, 0.5 mM δ-aminolevulenic acid and trace elements. The culture was incubated at 30°C in a bath with vigorous shaking; after 2.5 h isopropyl β-D-thiogalactopyranoside (1 mM) was added to induce the *tac* promoter and culture was maintained 24 h for the expression of pig CYP1A2 or 48 h for the expression of human CYP1A2.

CHAPS-solubilization of Escherichia coli-expressing pig and human CYP1A2

E. coli membranes containing pig or human CYP1A2 were solubilized as previously described (John et al. 1994). The culture cells were pelleted at 5000 rpm for 15 min at 4°C. The pellet was resuspended in 40 ml of MOPS buffer (100 mM MOPS pH 7.3, 10% glycerol, 0.2 mM dithiothreitol (DTT), 1 mM ethylenediamine tetra-acetic acid (EDTA)) and centrifuged as above. The pellet was again resuspended in MOPS buffer and sonicated several times with cycles of 15 s on and 5 s off. The sonicated sample was ultracentrifuged at 33 000 rpm for 30 min at 4°C. The pellet was suspended in 10 ml of MOPS buffer and stirred for 2 h at 4°C after the addition of CHAPS (0.5%). Finally, the sample was ultracentrifuged for 30 min at 4°C and the supernatant, containing the CHAPS-solubilized CYP1A2 preparation was stored at –80°C until use.

RT-PCR for tissue distribution studies

Total RNA (1 μg), extracted from the seven above-mentioned tissues of control pigs, after retrotranscription, was used for reverse transcriptase-polymerase chain reaction (RT-PCR) with a programme and primers for the 520 bp fragment previously described. The PCR products were analysed on agarose gel, purified and sequenced.

Immunoblot analysis

Microsomal proteins from liver (generally 10 μg) were separated according to Laemmli (1970) on sodium dodecylsulphate (SDS) 7.5% or 10% (w/v) polyacrylamide gel and then transferred electrophoretically onto nitrocellulose membranes following the method of Towbin et al. (1979). Immunodetection of CYP was performed either with anti-rat CYP1A1 (dilution 1 : 1000) or with anti-recombinant human CYP1A2 (dilution 1 : 2000) as the primary antibodies; the bands were visualized with a peroxidase-conjugated anti-rabbit IgG or anti-mouse IgG (dilution 1 : 1500). In all cases, in our experimental condition both antibodies recognized a single protein band in microsomes from either control or βNF-treated pigs. The intensity of protein bands was quantified by densitometry.

Purification of CYP1A2

The purification of porcine CYP1A2 was carried out in a sequence of steps (all performed at 4°C) similar to those previously described (Puccini et al. 1992). Microsomes obtained from a βNF-treated pig were resuspended in 0.1 M potassium phosphate buffer (pH 7.2) containing 1 mM EDTA, 1 mM DTT, 0.1 mM phenylmethanesulphonyl fluoride (PMSF) and 20% glycerol (solubilization buffer). They were solubilized with the addition of 0.6% sodium cholate for 30 min. The mixture was then centrifuged at 100 000 *g* and the supernatant was applied onto a *n*-octylamine-Sepharose 4B column (2.5 × 50 cm). After washing, the column was eluted with the same solubilization buffer containing 0.5% cholate and 0.05% emulgen. This fraction (fraction I) contained most of the CYP (1158 nmol) but not CYP1A1/2 as monitored by Western blot analysis with anti-rat CYP1A1. Fraction II, which contained CYP1A1/2, was eluted from the 4B column with the above solubilization buffer containing 0.5% cholate and 0.2% emulgen. This fraction II was concentrated by ultrafiltration (Amicon PM-30 membrane), extensively dialysed against 10 mM potassium phosphate pH 7.2, 1 mM EDTA, 20% glycerol, brought to 0.6% emulgen and loaded onto

a DEAE Sephacel anion-exchange column (1.6 × 35 cm) pre-equilibrated with this buffer. Most of the CYP (256 nmol) was recovered in the flow-through (DE-Sephacel fraction I) but it did not contain CYP1A1/2 and was left aside. The fraction enriched of CYP1A2 (DE-Sephacel fraction II) was eluted with the above buffer containing 50 mM NaCl, while a fraction containing CYP1A1 among other proteins was eluted with the same buffer containing 300 mM NaCl, and left. This chromatographic separation of CYP1A2 from CYP1A1 with DEAE-column by salt gradient was previously described in the separation of the corresponding isoforms from liver of treated rats (Ryan et al. 1980).

The DE-Sephacel fraction II after concentration and dialysis against 5 mM potassium phosphate pH 7.8, 1 mM EDTA, 20% glycerol, was loaded on a DE 53 column (1.6 × 15 cm), pre-equilibrated with this buffer and 0.6% emulgen. The fraction enriched of CYP1A2 was eluted with the same buffer containing 30 mM NaCl. This fraction was concentrated and dialysed against 10 mM potassium phosphate pH 6.8, 1 mM EDTA, 20% glycerol and loaded into a CM column (1.6 × 20 cm) pre-equilibrated with the same buffer and 0.5% emulgen. A fraction containing CYP1A2 as a single protein band, as judged by electrophoresis and Western blot, was eluted from this column by the above buffer plus 100 mM NaCl. The eluate from the CM was concentrated, dialysed against 10 mM potassium/phosphate pH 7.2, 1 mM EDTA, 20% glycerol and loaded into a small hydroxyapatite (HA) column (1.6 × 5 cm). After an extensive washing with the above buffer, containing 0.3% cholate to replace emulgen, the CYP1A2 was eluted with 0.25 M potassium phosphate pH 7.2 plus 0.3% cholate, concentrated and dialysed extensively (48–72 h) to remove cholate against 100 mM potassium phosphate pH 7.2, 1 mM EDTA, 20% glycerol and then stored at –80°C. Table I shows the results of a CYP1A2 purification procedure. Similar results were obtained when CYP1A2 was purified from liver microsomes of another βNF-treated pig. The identity and molecular mass of this protein was also determined by time-of-flight mass spectrometry (Protein Analysis Facility, Lausanne University, Lausanne, Switzerland), where an Mr of 58 kDa was obtained confirming that the purified protein was the porcine CYP1A2 devoid of a significant CYP1A1 contamination.

Enzyme assays

Cytochrome P450 content was measured by the method of Omura and Sato (1964).

Ethoxyresorufin *O*-deethylase (EROD) and methoxyresorufin *O*-demethylase (MEROD) activities were determined by measuring the production of resorufin, as previously reported (Lubet et al. 1985), with a Perkin-Elmer spectrofluorimeter (model LS 45). The rates of aniline hydroxylation (AnH) and of 6 β-testosterone hydroxylase (6 βT-H) were measured as previously described (Longo et al. 1991). Acetanilide hydroxylase (AcH) was determined

Table I. Purification of CYP1A2 from liver microsomes of a βNF-treated pig

Fraction	Total CYP (nmol)	Specific CYP content (nmol/mg prot.)	Yield
Solubilized microsomes	2865	0.68	100
Sepharose 4B column fraction II eluate	560	1.7	19
DE Sephacel fraction II eluate	92	3.3	3
DE 53 fraction I eluate	38	5.6	1.3
CM 0.1M NaCl eluate	15	8.7	0.5
HA 0.25 M K/phosphate eluate	8.4	11.2	0.3

by high-performance liquid chromatography (HPLC) as described by Tsyrlov et al. (1993). Caffeine oxidation was focused on the 3-demethylase (Caf-3D) activity using an HPLC method proposed by Agundez at al. (1992). 2-Aminofluorene hydroxylase (2-AmFH) activity was determined according to Vanderslice et al. (1987). The activities of the purified enzyme were determined, as described previously (Puccini et al. 1992), in a reconstituted system containing in about 0.3 ml, 0.1 nmol of CYP1A2, 0.3 nmol of rat NADPH-cytochrome P450 reductase, 30 μg of fresh sonicated dilaurylphosphatidylcholine which had been pre-incubated for 30 min at room temperature. Then, after the addition of phosphate buffer pH 7.4, the substrate and NADPH (1 mM) were added to a final volume of 1 ml, and the complete system was incubated for 20 min at 37°C. Under these conditions the catalytic activities were found to be optimal for all the tested substrates.

Kinetic parameters (K_m and V_{max}) for the EROD and MEROD fluorescent assays were investigated in a reconstituted system containing the 50 pmol of purified CYP1A2 or CHAPS-solubilized *E. coli* preparations of porcine CYP1A2 or human CYP1A2 (50 pmol), 100 pmol of rat NADPH-cytochrome P450 reductase and 30 μg of fresh sonicated dilaurylphosphatidylcholine. The reactions were performed as described before. Kinetic parameters were determined using non-linear regression analysis with GraphPad Prism software.

Inhibition experiments were performed to evaluate the sensitivity of pig and human recombinant CYP1A2. The inhibitory effects on EROD activity of α-naphthoflavone or ellipticine, two known inhibitors of human CYP1A2 (Tassaneeyakul et al. 1993), were examined. The assay method was carried out in a reconstituted system as previously described using a fixed substrate concentration (3 μM) and varying inhibitor concentrations. IC_{50} were estimated by interpolation.

Results

Purification of CYP1A2

In order to study the catalytic properties of porcine CYP1A2, we have purified this enzyme from the βNF-treated pigs by methods previously described (Ryan and Levin 1990; Puccini et al. 1992). The purification was performed from induced pigs, as the CYP1A2 content could be too low in the young control ones.

As reported earlier (Thomsen et al. 1991), the treatment of pig with βNF doubled the hepatic microsomal CYP content (from 0.31 to 0.62 nmol mg^{-1} protein in the control and treated animal, respectively).

As seen in Figure 1 and Table I, CYP1A2 has now been purified from microsomes to apparent homogeneity by various chromatographic steps. The specific content of the purified enzyme, which had an apparent Mr of about 57 kDa, was 11.2 nmol of CYP mg^{-1} protein, a value lower than that theoretically expected (about 16 nmol mg^{-1} protein) suggesting a significant presence of CYP (heme-free) apoprotein.

Spectral property of purified CYP1A2

The maximum absorbance of the reduced carbon monoxide complex of the purified CYP1A2 was at 448 nm, as previously reported by Thomsen et al. (1991). The absolute spectrum (Figure 2) of CYP1A2 had a Soret maximum at 393 nm, indicative of a predominant high-spin state as observed for other mammalian CYP1A2 (Guengerich 1997).

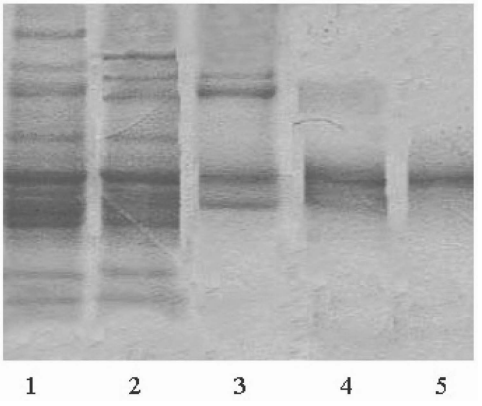

Figure 1. Gel electrophoresis of microsomal proteins at various stages of cytochrome P450 (CYP) 1A2 purification. Lane 1 contained solubilized microsomes from βNF-treated pig; lane 2, fraction II eluted from the DE Sephacel column; lane 3, fraction I eluted from the DE 53 column; lane 4, fraction eluted from the CM column; and lane 5, fraction eluted from the HA column.

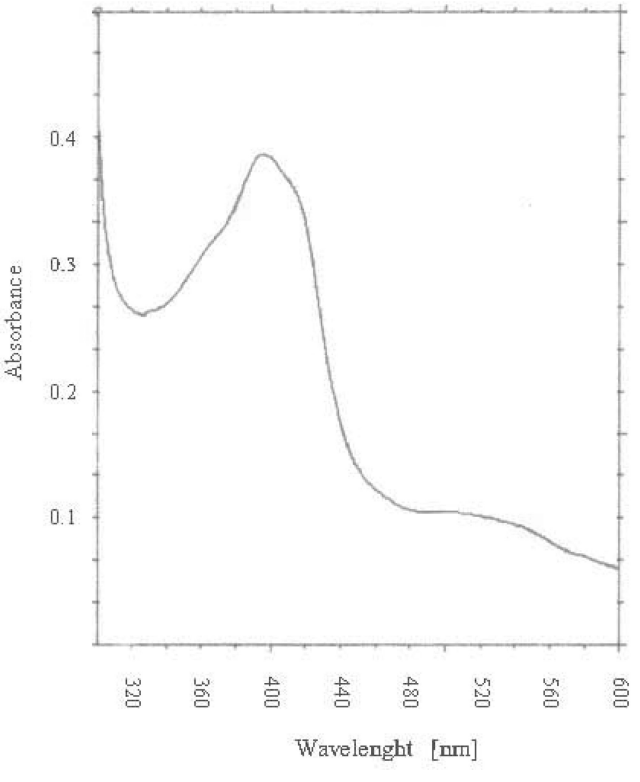

Figure 2. Absolute spectra of purified cytochrome P450 (CYP) 1A2 (3.4 μM) in 100 mM potassium phosphate buffer, pH 7.2, 20% glycerol, 0.1 mM ethylenediamine tetra-acetic acid (EDTA).

Table II. Catalyitc activities of CYP 1A2 and hepatic microsomes from a βNF-treated pig.

Substrate	Activity (nmol/min/nmol CYP)	
	Microsomes	CYP 1A2
Caf-3D	0.124	0.750
AcH	0.856	5.88
MEROD	0.043	0.174
EROD	0.776	0.575
AnH	0.211	N.D.
2-AmF-H	0.366*	0.353*
6βT-H	0.437	N.D.

The catalytic activities were measured as described in the Materials and Methods. Caf-3D, caffeine 3-demethylase, AcH, acetanilide hydroxylase, MEROD, methoxyresorufin O-demethylase; EROD, ethoxyresorufin O-deethylase; AnH, aniline hydoxylase; 2-AmFH, 2-aminofluorene hydroxylase; 6βT-H, 6β-testosterone hydroxylase, The data are the mean of duplicate experiments. *This activity is expressed as Δfluorescence/min/nmol CYP. N.D. = not detectable.

Figure 3. Western blotting of cytochrome P450 (CYP) 1A2 in hepatic microsomes from eight control pigs (lanes 1–8) using monoclonal antibodies anti-human CYP1A2. Lanes 9 and 10 contained, as a reference, 0.1 and 0.5 pmol of purified pig CYP1A2, respectively.

Drug oxidation activities

The oxidation activity of CYP1A2 towards a number of typical substrates for mammalian CYP1A2 (Lewis and Lake 1996) was compared, in a reconstituted monooxygenase system, with that of hepatic microsomes from βNF-treated pigs.

As shown in Table II, purified CYP1A2 is active on the oxidation of caffeine, acetanilide, methoxyresorufin, ethoxyresorufin and 2-aminofluorene. On the other hand, the oxidation of aniline or testosterone was not catalysed by porcine CYP1A2. The porcine enzyme catalysed more specifically the oxidation of caffeine, acetanilide and methoxyresorufin: their rates were about four- to six-fold higher in the reconstituted system than in the βNF-treated microsomes.

Immunodetection analysis

To evaluate the constitutive expression of CYP1A2, we determined two marker activities (MEROD and AcH) and performed immunoblotting on hepatic microsomes prepared from eight control pigs. The monoclonal antibodies raised against human CYP1A2 detected in the liver microsomes of all pigs a single band (Figure 3) whose interindividual variation correlated positively with the activities of MEROD ($r = 0.92$) and of AcH ($r = 0.58$) but not with those of EROD ($r = 0.27$). The hepatic protein band recognized by these antibodies should only account for the expression of CYP1A2 as these antibodies do not cross-react

with human CYP1A1. However, both the immunoblots and marker activities revealed an interindividual variation in the expression of CYP1A2 in porcine liver. Quantitative Western blots, using purified CYP1A2 concentrations as the standard curve, showed that CYP1A2 varied from approximately 3% to 9% of the total CYP present in pig liver microsomes.

Cloning and nucleotide sequencing of porcine CYP1A2

Oligonucleotide primers for RT-PCR were designed on the base of the conserved regions of the mammalian CYP1A2 isoforms retrieved from GeneBank. A 520 bp PCR product was obtained and, after sequencing, it resulted in being highly homologous to human CYP1A2. Nucleotides in the 5′- and 3′-regions of the cDNA were obtained by using the 5- and 3-RACE procedures. The sequence of the full-length cDNA, which was 1827 bp (Figure 4), contained a 69-nucleotide 5′-UTR, an open reading frame of 1548 bp and a 210 bp 3′-UTR. The cDNA encoded a protein of 516 amino acids with an estimated molecular weight of 58 380 Da. This sequence was subsequently submitted to GeneBank and then compared with those of other animals.

CYP1A2 of pig had a high degree of nucleotide and amino acid sequence identity with the reported sequences for the CYP1A2 of other mammals (Table III), but, as expected, shared a lower amino acid identity (76%) with the pig CYP1A1 (GeneBank accession number NM 24412). The porcine CYP1A2 gene appeared to be more similar to the corresponding genes of *Bos taurus* (accession number XM 59450) *Balaenoptera acutorostrata* (accession number AB 231892), *Homo sapiens* (accession number NM 012541), *and Macaca fascicularis* (accession number D 86474) than those of *Canis familiaris* (accession number NM 001008720) or *Rattus norvegicus* (accession number NM 012541). The N-terminal sequence of CYP1A2 contained approximately 20 hydrophobic amino acids preceding by a halt-transfer signal containing positively charged amino acids and a proline rich region (residues 42–54), necessary for proper protein folding (Kemper 2004). CYP1A2 possessed a putative heme-binding domain (residues 451–460) around Cys^{458} that was well conserved among the mammalian CYP1A2 proteins (Figure 4). The sequences alignment of the putative substrate recognition sites of pig CYP1A2 and those of mammalian orthologues showed a degree of highly conserved amino acids which ranked in the following order: *Macaca fascicularis* > *Bos taurus* > *Homo sapiens* > *Canis familiaris* > *Balaenoptera acutorostrata* ≥ *Rattus norvegicus* (Table IV). The terminal nucleotide sequence of 3′-UTR, although incomplete, showed no identity with those of human or rat CYP1A2s.

Table III. Nucleotide and amino acid sequence identity between CYP1A2 of pig and the CYP1A2 of other mammals.

Species	Nucleic acid % identity	Amino acid	
		% Identity	% Similarity
Bos taurus	87	82	91
Balaenoptera acutorostrata	88	82	90
Homo sapiens	85	81	90
Macaca fascicularis	85	81	89
Canis familiaris	83	77	87
Rattus norvegicus	78	73	85

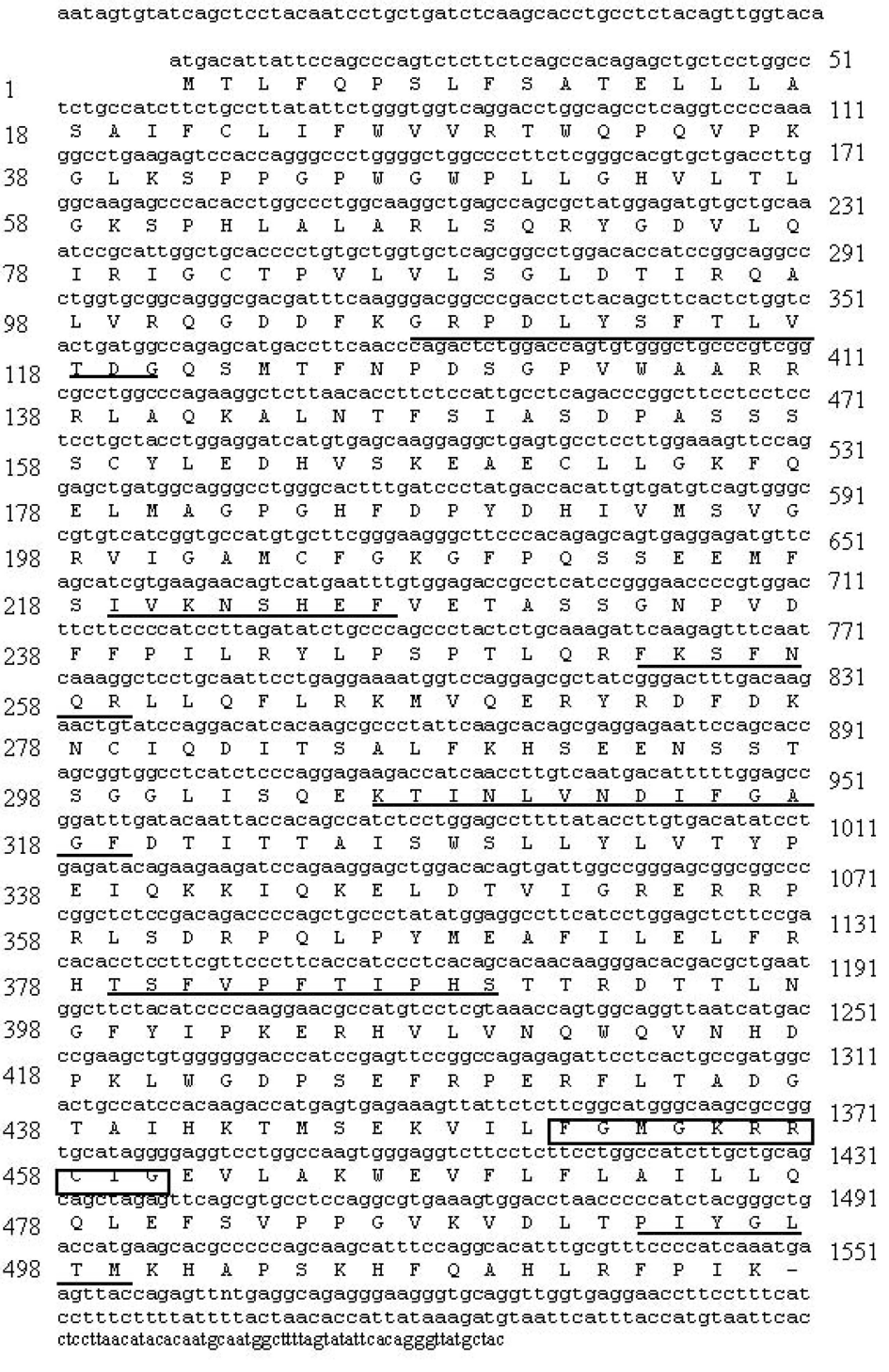

Figure 4. Nucleotide and deduced amino acid sequence of the pig cytochrome P450 (CYP)1A2. The putative initial ATG start codon, the TGA stop codon and the observed mutant nucleotides are emboldened; the haem region is boxed, and the six putative SRS regions are underlined.

Table IV. Sequence comparison between swine CYP1A2 and other mammalian orthologues at substrates recognition sites (SRS 1-6) and heme binding domain

Domain	Sus scrofa aa residues in CYP1A2	*Homo sapiens* CYP1A2	*Macaca fascicularis* CYP1A2	*Balaenoptera acutorostrata* CYP1A2	*Bos taurus* CYP1A2	*Canis familiaris* CYP1A2	*Rattus norvegicus* CYP1A2
SRS1	107-130	75	83	95	100	87	87
SRS 2	218-228	80	93	55	55	33	55
SRS 3	252-260	80	80	40	93	93	55
SRS 4	305-321	87	87	93	81	93	75
SRS 5	379-390	87	93	93	100	100	100
SRS 6	491-500	100	100	93	93	100	100
HEME	451-460	100	90	100	100	100	90

Percentage of identity with the pig sequence.

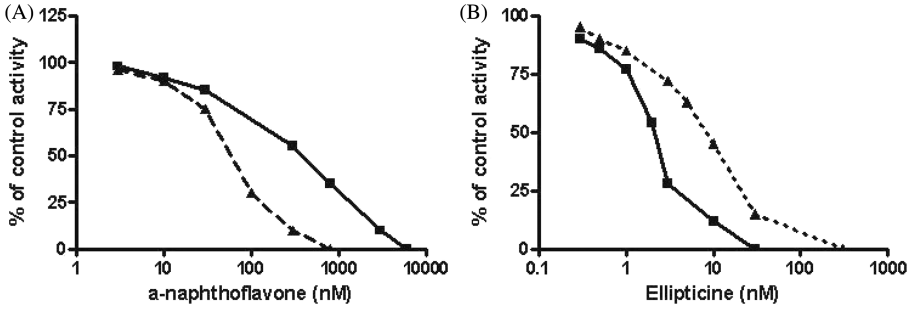

Figure 5. 7-Ethoxyresorufin *O*-deethylase (EROD) assay (3 μM substrate concentration) was performed with pig (■) or human (▲) recombinant cytochrome P450 (CYP) 1A2 in a reconstituted system and inhibitor concentrations of α-naphthoflavone (A) and ellipticine (B). Values, expressed as percentages of control activity, are the mean of two experiments which differ by less than 8%.

Mutation analysis of porcine CYP1A2

In order to identify any prominent genetic polymorphism of CYP1A2 in pigs, PCR analysis was performed in the liver of eight animals. Sequences analysis detected a polymorphism in five coding regions: at protein position 198 (gca → gcg), 211 (cgc → tgc), 231 (caa → ccg), 328 (aac → gac) and 402 (gct → gcc).

These mutations, except those in positions 211 and 328, are silent mutations since they do not result in a different amino acid sequence. In contrast, a mutation at position 211 (detected in four animals out of eight) caused an amino acid change from R to C, and, likewise, a mutation at position 328 (detected in two animals out of eight) caused a replacement D with N, an amino acid known to be present in other mammalian CYP1A2.

Porcine and human CYP1A2 enzymes expressed in Escherichia coli.

The porcine and human CYP1A2 were expressed separately in *E. coli* DH5α cells. Both CHAPS-solubilized pig and human CYP1A2 preparations from *E. coli* showed the reduced carbon monoxide difference spectra with a predominant absorption peak at 448 nm and a little peak at 420 nm (spectra not shown). These results indicated that both pig and human CYP1A2 were expressed as the holo-enzymes. Based on these difference spectra, the

Table V. Kinetic parameters of EROD and MEROD activities with recombinant pig and human CYP1A2, hepatic microsomes from control pig and CYP1A2 purified from βNF-treated pig.

	EROD		MEROD	
Parameters	Km (μM)	V_{max}⋆	Km (μM)	V_{max}⋆
Recombinant pig 1A2	0.41	0.035	3.1	0.013
Recombinant human 1A2	0.85	0.090	2.6	0.007
Pig liver microsomes	0.64	0.008	2.2	0.001
1A2 purified from pig liver	0.34	0.712	1.8	0.218

The incubations were performed as reported in the Material and Methods. Substrate concentrations ranged from 0.1 to 10 μM. Values are the mean of two experiments that differ less than 23%. ⋆values expressed as nmol/min/nmol P450.

CHAPS-solubilized preparations yielded about 17.4 nmol of pig CYP1A2 and 13.6 nmol of human CYP1A2 per litre of culture, respectively.

EROD and MEROD kinetic assays

EROD and MEROD assays were performed using microsomes from control pigs, CYP1A2 purified from liver, solubilized *E. coli* membrane preparations containing pig CYP1A2 and were compared with *E. coli* preparations containing human CYP1A2 (Table V). The apparent K_m values for both reactions either relative to pig CYP1A2 or to human counterpart showed a high degree of similarity. Furthermore, in all systems the pig CYP1A2 showed a lower K_m for EROD compared with that for MEROD, as previously reported for the human orthologous (Kim et al. 2008). On the other hand, the apparent V_{max} values showed differences among the various enzymatic reactions. As expected, the apparent V_{max}'s for both EROD and MEROD of pig recombinant CYP1A2 were higher than those obtained with pig liver microsomes but lower than those of CYP1A2 purified from pig liver.

It is of interest to note that the apparent V_{max} value of recombinant pig CYP1A2 for MEROD, unlike EROD, was higher than that of recombinant human CYP1A2 suggesting that this activity may be a better marker for this enzyme in pig than in human.

Chemical inhibition assays

Inhibition properties of pig and human CYP1A2 by α-naphthoflavone and ellipticine were determined using CHAPS-solubilized recombinant enzymes in a reconstituted system. The effects of α-naphthoflavone and ellipticine on EROD activity catalysed by pig and human CYP1A2 were examined (Figure 5). In both cases, the best inhibitor was ellipticine. The IC_{50} value for α-naphthoflavone was eight times lower for recombinant human CYP1A2 (55 nM) compared with pig counterpart (450 nM). Conversely, the IC_{50} value for ellipticine was five-fold lower for pig CYP1A2 (2.5 nM) compared with human counterpart (9.2 nM).

Tissue distribution of CYP1A2

The tissue distribution of CYP1A2 mRNA in the pig was analysed by RT-PCR using specific primers. As shown in Figure 6, an intense band corresponding to the size of the

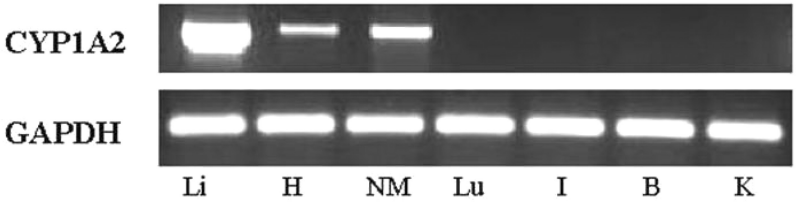

Figure 6. Reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of cytochrome P450 (CYP) 1A2 messenger RNA (mRNA) in various organs of control pigs: liver (Li), heart (H), nasal mucosa (NM), lung (Lu), intestine (I), brain (B), and kidney (K). Polymerase chain reaction (PCR) products were separated by electrophoresis on agarose gel and stained with ethidium bromide.

expected amplified region (520 bp) was obtained using the total RNA from liver. A weak band of the same size was also observed with the total RNA from heart and nasal mucosa, whereas no bands were seen with the total RNA from lung, small intestine, kidney and brain. Specific primers for GAPDH were used as a control for RT-PCR experiments.

Discussion

In previous works, the cytochrome P450 (CYP) 1A2 gene and enzyme have been characterized in a number of mammalian species, but similar studies have so far been missing in pigs (Guengerich 1997; Sakuma et al. 1997; Tanaka et al. 2006). We have now isolated the CYP1A2 enzyme from β-naphthoflavone (βNF)-treated pigs, characterized some of its biochemical properties and cloned the coding DNA (cDNA) encoding this enzyme. Using methods similar to those employed in the isolation of other mammalian CYPs (Ryan and Levin 1990; Marini et al. 1998), we have purified to apparent homogeneity the porcine CYP1A2, which, as previously reported (Thomsen et al. 1991), was obtained predominantly in the high-spin state. As expected, the purified protein had catalytic properties common to those of other CYP1A2 enzymes; it was found to be catalytically very active towards the typical substrates of CYP1A2 enzymes such as caffeine, acetanilide, methoxyresorufin, ethoxyresorufin and 2-aminofluorene. In particular, the catalytic activities of the purified protein towards caffeine, methoxyresorufin and acetanilide, all known marker substrates for CYP1A2 enzymes (Lewis and Lake 1996), were markedly increased over those found with liver microsomes from βNF-treated pigs. Interestingly, the purified pig enzyme, unlike rat CYP1A2 (Ryan and Levin 1990), failed to oxidize aniline or testosterone (in the 6 β-position), a property shared with human CYP1A2 (Waxman et al. 1991; Ono et al. 1996), thereby indicating a closer substrate specificity between pig and human enzymes than between rat and human counterparts.

We have also studied the expression of CYP1A2 in the hepatic microsomes of control pigs, and found a positive correlation between the results of immunoblot analysis and those of selective CYP1A2 activities (methoxyresorufin *O*-demethylase (MEROD) and acetanilide hydroxylase (AcH)) suggesting that this isoform is expressed constitutively in pig liver and that the antibodies employed can only recognize this enzyme. More work is, however, necessary to exclude conclusively that these antibodies can also recognize, in addition to CYP1A2, any constitutively expressed CYP1A1, which has an Mr similar to that of CYP1A2 (Thomsen et al. 1991). In human liver, CYP1A2 accounts for about 10–13% of the total

CYP content (Rendic and Di Carlo 1997) and is essentially the only constitutively expressed enzyme of CYP1A subfamily. Assuming that this also applies to the livers of our young male domestic pigs, the constitutively expressed CYP1A2 isoform was found to be 3–9% of the total CYP content and showed a significant interindividual variation.

In order to characterize further this porcine enzyme, we isolated and cloned the cDNA of CYP1A2 by using a polymerase chain reaction (PCR) approach followed by 3′- and 5′-rapid amplification of cDNA ends (RACE). The CYP1A2 cDNA isolated in this study was 1827 bp long and contained an open reading frame of 1548 bp encoding a protein of 516 amino acids and the 5′- and 3′-non-coding regions. The number of amino acids of the porcine protein is the same as that reported for human CYP1A2 and that of the common minke whale, and several monkey species (Niimi et al. 2005; Narimatsu et al. 2005) but different to that reported for dog, cat and rodents (Uchida et al. 1990; Lewis and Lake 1996; Tanaka et al. 2006). When compared with other CYP1A2 enzymes across species, the porcine gene and its substrate recognition sites (Gotoh 1992) shared a higher identity with the sequences of monkey, human and bovine CYP1A2 than with those of dog or rodents.

The degree of identity between pig and human CYP1A2 varies according to the substrate recognition sites (SRS) considered, going from a maximum of 100% for SRS6 to a minimum of 75% for SRS1, a region latterly involved in the flexible channel of substrate entry into the active site cavity. The comparison between the overall amino acid composition of porcine CYP1A2 with that of human CYP1A2 demonstrated that most differences represented conservative changes, i.e. involved replacement of amino acid residues with others possessing similar chemical properties.

A more detailed comparison of the amino acid sequence of pig CYP1A2 with that of human protein, which takes into account the recently resolved human structure (Sansen et al. 2007), revealed specific motives of interest.

Among the amino acid residues F226, Thr223, Asp320, Tyr189, Val220, Thr498 and Lys500, which participate in an extensive network of hydrogen-bonded water and side-chains in the active site of human CYP1A2, only Thr223 was not shared by the porcine counterpart where Ser223 was found.

The amino acids Thr118, Ser122 and Thr124 that characterize the B′-region (SRS1) of the human CYP1A2 structure were all shared by the porcine enzyme. A significant difference was found in amino acid 322, which was Val in pig and Leu in human. This variation is thought to be important for modifying the catalytic preference of CYP1A2 and CYP1A1 with respect to MEROD and 7-ethoxyresorufin *O*-deethylase (EROD), respectively (Liu et al. 2004).

Due to the use of domestic hybrid pigs, various single nucleotide polymorphisms (SNPs) ($n = 5$) were found in the sequence analysis of the CYP1A2 gene, even though a small number of animals ($n = 8$) were used. However, of the five differences of nucleotides observed, only two resulted in a change of amino acid (R 71 C and D 110 N). In this regard, it is of interest to note that amino acid R 71 is shared with human and marmoset but not with cynomolgus and Japanese monkeys or other mammals (Lewis and Lake 1996; Narimatsu et al. 2005). Of course, the influence of these different amino acids on the activity of CYP1A2 remains to be examined. However, in the present study, which was limited to only a few pigs, no important mutations including non-sense mutations have been brought to light, as reported in dogs (Mise et al. 2004).

To express pig CYP1A2 in *Escherichia coli*, a construct was prepared with the modification of seven codons in the 5′ terminal of CYP1A2 cDNA as reported by Fisher et al. (1992) and examined for the expression in the pCW vector. The present results support the well-demonstrated utility of this N-terminal modification on the expression of CYP in *E. coli*.

Indeed, the expression of porcine CYP1A2 was completely dependent upon modification of the *N*-terminal. With the optimization with respect to the induction time (24 h) and temperature (30°C), 15–18 nmol of CYP1A2 l^{-1} of culture were routinely recovered in the *E. coli* membranes, a yield similar to that found for the expression of human CYP1A2. The 3-[(3-cholamidopropyl)-dimethylamonio]-1-propanesulfonate (CHAPS)-solubilized *E. coli* preparations of pig and human CYP1A2 were used to make a comparison of their catalytic properties. In the EROD and MEROD assays, it was found that the K_m's of these two enzymes are very similar suggesting that pig and human CYP1A2 have practically the same affinity for these reactions. However, the V_{max}'s of these enzymes for these oxidations showed differences indicating that pig CYP1A2 catalyses the MEROD better than the human counterpart. Thus, in agreement with correlation coefficient data, this activity may be a useful marker for the CYP1A2 isoform in pig.

Inhibition experiments further suggested a certain difference of the biochemical properties between pig and human CYP1A2 enzymes. Compared with the results obtained, ellipticine was a better inhibitor of EROD activity catalysed by pig CYP1A2 rather than human orthologue, whereas the reverse was found for α-naphthoflavone.

We have also examined the relative tissue distribution of CYP1A2 in pig and found that the enzyme was mainly expressed in the liver, although it was also detected, at least at the mRNA level, in heart and nasal mucosa. This predominantly hepatic expression is in agreement with what has been reported in humans and some other mammals (Lewis and Lake 1996; Guengerich 1997).

The results obtained in the present study indicate that the biochemical properties of the purified porcine CYP1A2 are considerably similar to those of the corresponding human enzyme. From the analysis of cloned porcine CYP1A2 cDNA, it was observed that the nucleotide and deduced amino acid sequences had 85% and 81% identities to those of the human counterparts, respectively. A high degree of similarity was also found among the SRSs of the porcine and human CYP1A2.

In conclusion, taken together these results suggest that hepatic CYP1A2 in pig and human may have a similar function in the biotransformation of endobiotic and xenobiotic compounds. The availability of an adequate amount of porcine CYP1A2, which has never been expressed in a heterologous system, can now facilitate a systematic investigation of the substrate specificity of drugs on development. The evaluation of toxicity and metabolism of new drugs is based on experiments with animal systems and, therefore, the selection of an appropriate animal model such as the pig is of great importance. This investigation extends our knowledge of the CYP-dependent drug-metabolizing system in pig and contributes to the idea that this animal is a useful human model in toxicological and pharmacological studies.

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