# Chlorzoxazone Metabolism by Porcine Cytochrome P450 Enzymes and the Effect of Cytochrome b<sub>5</sub>

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## ABSTRACT:

RUG METABOLISM & DISPOSIT

Chlorzoxazone (CLZ) is a commonly used nontoxic in vivo and in vitro probe for the assessment of CYP2E1 activity. Human CYP1A1 and CYP3A4 have also been shown to contribute to CLZ metabolism. For pigs to be a potential model system for humans, it is necessary that human and pig cytochromes P450 (P450) have similar metabolizing capabilities. Therefore, CLZ metabolizing capabilities and specificities of porcine P450s were investigated. In this study, the complete coding regions of six porcine P450s were amplified from liver cDNA and cloned into pcDNA3.1/V5-His TOPO vector. Expression vectors for the individual P450s and microsomal cytochrome  $b_5$  (CYB5A) were expressed in the human embryonic kidney HEK-293*FT* cell line to investigate their role in CLZ metabolism. As with the human enzymes, porcine CYP2E1 ( $K_m = 290.3 \mu M$  and  $V_{max} = 4980$  pmol/h/mg total protein) and CYP1A1 ( $K_m = 159.5 \ \mu$ M and  $V_{max} = 1650 \ pmol/h/mg$  total protein) both contribute to CLZ metabolism. In addition, porcine CYP2A19 and CYP2C33v4 also metabolize the substrate, with  $K_m = 212.1 \ \mu$ M and  $V_{max} = 6680 \ pmol/h/mg$  total protein and  $K_m = 126.3 \ \mu$ M and  $V_{max} = 2100 \ pmol/h/mg$  total protein, respectively, whereas CYP3A does not. CYB5A augmented CYP2E1 and CYP2C33v4 activity in the pig, with a significant increase in activity of 85 and 73% compared with control, respectively. Thus, CLZ should be used with caution as a probe for CYP2E1 activity in the pig. However, further information regarding the abundance of different P450 isoforms is needed to fully understand their contribution in microsomal, hepatocyte, and in vivo systems in the pig.

The CYP2E1 gene is the most highly conserved of all P450s and is found in numerous species including human, mouse, rat, pig, dog, cow, rabbit, monkey, and even fungi (Overton et al., 2008). This enzyme shows broad substrate specificity with more than 80 substrates identified, most of them being small, hydrophobic compounds, including anesthetics (Lieber, 1997; Ingelman-Sundberg, 2004). There is much interest in exploring the metabolic capabilities of human CYP2E1, because it contributes to the metabolism of many compounds ranging from therapeutic agents to procarcinogens and carcinogens (Raucy et al., 1993; Lieber, 1997). In addition, CYP2E1 expression can be altered by growth hormone, cytokines, ethanol, thyroid hormone, and insulin (Waxman et al., 1989; Abdel-Razzak et al., 1993; Lieber, 1997; Peng and Coon, 1998; Emery et al., 2003). Because of differences in CYP2E1 expression, individuals with diabetes, endocrine disorders, or alcoholism may exhibit alterations in drug metabolism and efficacy (Overton et al., 2008).

Characterization of the metabolic capabilities of an enzyme usually involves the use of specific inhibitors and probe substrates as indicators of enzymatic activity. Chlorzoxazone (CLZ), a centrally acting

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muscle relaxant, was first shown to be metabolized by human CYP2E1 by Peter et al. (1990) and has since been used as a specific probe substrate in both in vitro and in vivo studies. One of the challenges of choosing probe substrates, apart from sensitivity and specificity, is that they must be relatively nontoxic to be suitable for use in in vivo studies; CLZ meets this criterion (Court et al., 1997; Ernstgård et al., 2004). However, the specificity of CLZ as a probe substrate for human CYP2E1 has been questioned, because other human P450s contribute to its metabolism (Carriere et al., 1993; Gorski et al., 1997). Just as in the human, porcine CYP2E1 has been shown to metabolize CLZ to its major metabolite, 6-OH-CLZ (Anzenbacherová et al., 2005; Baranová et al., 2005).

Pigs are often explored as potential human models in pharmaceutical studies because of their wide availability and physiological, dietary, and anatomical similarity to humans (Balk, 1987; Monshouwer et al., 1998; Tsiaoussis et al., 2001). Drug development is a complex process requiring multiple steps before clinical trial testing. Individual porcine P450 enzymes have been studied to determine how metabolic capabilities correlate with those found in humans and in veterinary pharmacology (Juskevich, 1987). Studies have demonstrated that porcine hepatocytes and microsomal systems are capable of metabolizing a wide range of human indicator substrates (Bogaards et al., 2000; Myers et al., 2001; Szotáková et al., 2004). CLZ has also been used as a probe for porcine CYP2E1activity in pharmaceutical and agricultural studies (Friis, 1995; Lejus et al., 2002), but as is the Downloaded from dmd.aspetjournals.org at ASPET Journals on April 29,

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**ABBREVIATIONS:** P450, cytochrome P450; CLZ, chlorzoxazone; 6-OH-CLZ, 6-hydroxy-chlorzoxazone; HEK293*FT*, human embryonic kidney cell line; POR, P450-oxidoreductase/P450 NADPH reductase; CYB5R3, cytochrome  $b_5$  reductase; CYB5A, microsomal cytochrome  $b_5$ ; h, human;  $C_{int}$ , intrinsic clearance.

case in humans, it is highly likely that other porcine P450 enzymes contribute to CLZ hydroxylation. It is important to determine which porcine enzymes may have a significant contribution to CLZ metabolism if the pig is to be an effective human model. If CLZ is used as a marker for CYP2E1 activity, the potency of CYP2E1 inhibitors may be underestimated in pharmacological studies if other P450s contribute to CLZ hydroxylation. Commonly used anesthetics are metabolized by CYP2E1 and some, such as propofol, are inhibitors of CYP2E1 (Lejus et al., 2002). In addition, the microsomal hemoprotein cytochrome  $b_5$  (CYB5A) has been shown to augment human CYP2E1 activity toward CLZ hydroxylation by apparently acting as an electron donor (Yamazaki et al., 1996, 2002).

Most studies use human probe substrates, inhibitors, or antibodies to characterize P450 enzymes in other species, and this approach assumes that these compounds are also specific to the particular porcine isoforms of the human enzymes. However, this is often not the case. Therefore, to definitively characterize the porcine P450 isoforms that may contribute to CLZ metabolism, we have cloned and individually expressed various porcine P450s in a cell line system, eliminating the need for inhibitors and antibodies to identify which isoforms are present. In this study, we investigated the specificity of CLZ metabolism by individually expressing porcine CYP2E1, CYP1A1, CYP2A19, CYP2C33v4, CYP2C49, and CYP3A as well as human CYP2E1 in human embryonic kidney (HEK-293FT) cells along with porcine P450-oxidoreductase. We measured the apparent clearance efficacy of CLZ by each enzyme in our model system. We also investigated the effect of cotransfection with an expression vector for CYB5A on the rate of CLZ hydroxylation, because little is known about the effects of CYB5A on the activity of individual porcine P450s.

#### Materials and Methods

Cloning. The expression vectors for porcine P450-oxidoreductase/P450 NADPH reductase (POR), cytochrome  $b_5$  reductase (CYB5R3), and CYB5A were constructed as described previously (Billen and Squires, 2009). To generate expression vectors for the different porcine P450s, the entire coding regions of porcine CYP1A1, CYP2A19, CYP2E1, CYP2C33v4, CYP2C49, and CYP3A were amplified from porcine liver cDNA by polymerase chain reaction using platinum Taq DNA Polymerase High Fidelity (Invitrogen, Burlington, ON, Canada). The amplicons were then T/A cloned into pcDNA3.1/V5-His TOPO (Invitrogen) according to the manufacturer's instructions. PCR conditions included an initial denaturation step for 2 min at 94°C, 30 s at 94°C, and annealing of primers for 15 s at 60.1°C with an extension for 1 min 40 s at 68°C for 35 cycles. A final extension of 10 min was carried out at 68°C. Primers were designed based on their respective sequences available from National Center for Biotechnology Information; details are presented in Table 1. A plasmid containing human (h) CYP2E1 was obtained from Origene (Rockville, MD), and the coding region was amplified and cloned into pcDNA3.1/V5-His TOPO (Invitrogen). Expression vectors for V5-His tagged proteins were generated so that the levels of the expressed proteins could be estimated by Western blotting using anti-V5 antibody; the introduction of the V5-His tag has been shown to not interfere with enzymatic activity (Handley-Gearhart et al., 1994; Billen and Squires, 2009). The identity of all clones was confirmed by sequencing. The amplified sequence of porcine CYP3A did not match the sequence of porcine CYP3A29, differing by two amino acids: Asn423→His and Lys458→Arg. To ensure that this was not a cloning artifact, the sequence of CYP3A was confirmed by identifying its presence in liver cDNA prepared from three different breeds of pigs (Yorkshire, Large White, and Sireline).

Cell Culture. HEK-293FT cells (Invitrogen) were cultured in 75-cm flasks (Sarstedt, Montreal, QC, Canada) under conditions described previously (Billen and Squires, 2009). Cells were plated at 10<sup>6</sup> cells/well in six-well plates (BD Biosciences, Mississauga, ON, Canada). Confluent cells were transfected with Lipofectamine 2000 (Invitrogen) 24 h after plating. Each transfection contained 2.35  $\mu$ g of individual P450 expression plasmid along with 0.40  $\mu$ g of POR, 0.25 µg of CYPB5R3, and 1 µg of CYB5A. When plasmid amounts were varied, empty pcDNA3.1/V5-His TOPO vector was used to bring the total DNA amount up to 4.0 µg for each transfection. Twenty-four hours later, transfected cells were subsequently incubated with various amounts of CLZ (Sigma-Aldrich, St. Louis, MO) dissolved in dimethyl sulfoxide; the concentration of organic solvent did not exceed 0.1% in culture. Substrate was incubated for 24 h and a 1-ml aliquot of media was collected for analysis; trials were performed in triplicate. Protein was precipitated from the collected media by the addition of 1/10 volume of trichloroacetic acid (40% v/v) and centrifuged for 15 min; 100  $\mu$ l of supernatant was then subjected to HPLC analysis. Cells were scraped into 300 µl of radioimmunoprecipitation assay buffer containing 0.5% sodium deoxycholate, 1% SDS, and 1% Nonidet P-40 (Sigma-Aldrich) with a Complete protease inhibitor tablet (Roche, Mississauga, ON, Canada), sonicated for 1 min, and then centrifuged to pellet cell debris. The protein concentration was measured using the Bio-Rad protein assay (Bio-Rad Laboratories, Richmond, CA).

Western Blot. Western blot analysis was performed as described previously (Billen and Squires, 2009) with the following modifications. Proteins were separated on SDS-12% polyacrylamide gels and incubated with a 1:5000 dilution of primary mouse-anti-V5 antibody (Invitrogen) and a 1:24,000 dilution of secondary goat-anti-mouse-horseradish peroxidase antibody (Sigma-Aldrich). The blot was visualized as described previously (Billen and Squires, 2009), and band densities were analyzed with Northern Eclipse software (Empix Imaging, Mississauga, ON, Canada). Because all proteins contained the V5-His tag, immunostaining with anti-V5 antibody allowed measurement of all expressed P450s in individual samples. To ensure that the activity in each sample was based on equal protein content of the individually expressed P450s based on immunoblot band densities.

**HPLC Assays.** A 100- $\mu$ l volume of sample was analyzed by HPLC on a C18 reverse-phase column (250 × 4.6 mm, 5  $\mu$ m). The solvent system consisted of buffer A (5% acetonitrile, 95% water, and 0.2% acetic acid) and buffer B (100% acetonitrile) with a flow rate of 1 ml/min. The gradient used was as follows: 0 min at 80% A, 10 min at 10% A, 15 min r 10% A, 15.1 min at 80% A, and 20 min at 80% A. CLZ and the 6-OH-CLZ metabolite were monitored by absorbance at 295 nm; the retention times for 6-OH-CLZ and CLZ were 6.4 and 9.45 min, respectively.

**Data Analysis.** Statistical analysis was performed using SAS/STAT (version 9.1; SAS Institute, Cary, NC). The CYB5A dose response was

## TABLE 1

Summary of	primers	used f	for PCR	amplification	of CY	P450 isoforms
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Forward primers were modified by adding a Kozak sequence (GCC) immediately upstream of the start codon (underlined). Reverse primers were designed to eliminate the original stop codon of the protein sequence allowing for the incorporation of the V5-His-tag.

Protein	Forward (5'-3')	Reverse (5'-3')	Plasmid Name	Reference Sequence
Human CYP2E1 Porcine CYP1A1 Porcine CYP2C33v4 Porcine CYP2C49 Porcine CYP2A19 Porcine CYP3A	GCCATGTCTGCCCTCGGAGTC GCCATGTTCTCTGTGTTTGGA GCCATGGAGCTGCTGGGAATC GCCATGGATGGGGGCTGTGGT GCCATGCTGGCCTCAGGC GCCATGGACCTGATCCCA	TGAGCGGGGAATGACACAGAG AGAGCGCACATGCATCTGGAC TCTAGGGATGAGGCACAGCTT TACAGGAATGAAGCGGAGCTG GCGGGGCTGGAAGCTCATGGT GGCTCCACTTACGGTCCCATC	hCYP2E1 CYP1A1 CYP2C33v4 CYP2C49 CYP2A19 CYP3A	NM_000773.3 NM_214412 NM_214414 NM_214414 NM_214420 NM_214420 NM_214417 NM_214423
Porcine CYP2E1	GCCATGACTGCCCTGGGC	CACTTGTGAGCGGGGGAATGAC	CYP2E1	AB052259

analyzed using linear regression. All kinetic parameters were determined using SigmaPlot 8.0 (Systat Software, Inc., San Jose, CA) with Kinetic Module software. The  $K_m$  and  $V_{max}$  parameters were determined from either the Michaelis-Menten equation (eq. 1) or the Hill equation (eq. 2); examination of the Eadie-Hofstee plot was used to determine the most appropriate model. When a "hook"-shaped as opposed to a linear Eadie-Hofstee plot was observed, the data were fitted using the Hill equation because this is characteristic of sigmoidal data (Madan et al., 2002).

$$\nu = \frac{V_{\text{max}} \cdot [S]}{K_{\text{m}} + [S]} \tag{1}$$

$$\nu = \frac{V_{\max} \cdot [S]^n}{K' + [S]^n} \tag{2}$$

#### Results

An intact cell system was used to assess the activity of the individual P450 isoforms (CYP1A1, CYP2A19, CYP2E1, hCYP2E1, CYP2C33v4, CYP2C49, and CYP3A) toward CLZ. Initial transfections consisted of 0.40, 0.70, 0.25, and 1 µg of POR, P450, CYB5R3, and CYB5A plasmid amounts, respectively, because similar band intensities were obtained for each protein on Western blotting. Typical Western blots obtained from individual transfections are depicted in Fig. 1. Pretrials were conducted to determine which P450s were active in catalyzing CLZ hydroxylation with a CLZ concentration of 400 µM. Porcine CYP3A did not produce detectable metabolites and CYP2C49 produced 10-fold lower metabolite levels than the other P450s; therefore, these P450s were not chosen for further analysis. The production of 6-OH-CLZ was linear with increasing amounts of P450 expression plasmids that were transfected and was linear up to 72 h of incubation (data not shown). Control incubations of cells without P450 plasmid but transfected with POR, CYB5R3, and CYB5A and treated with CLZ did not produce any detectable 6-OH-CLZ metabolite (data not shown).

Chlorzoxazone was incubated at 25 to 700, 50 to 900, and 50 to 700  $\mu$ M for transfections with hCYP2E1, porcine CYP2E1, and CYP1A1, respectively (Fig. 2). The production of 6-OH-CLZ by these P450s displayed sigmoidal kinetics, and the data were therefore modeled by the Hill equation. For transfections with CYP2C33v4 and CYP2A19, chlorzoxazone was incubated at 10 to 600 and 25 to 800  $\mu$ M, respectively (Fig. 3). The production of 6-OH-CLZ by these P450s was modeled by the Michaelis-Menten equation. The kinetic parameters  $K_{\rm m}$  and  $V_{\rm max}$  along with the  $V_{\rm max}/K_{\rm m}$  ratio [intrinsic clearance, are summarized in Table 2.

The effect of CYB5A on CLZ metabolism was next investigated by separate cotransfections of increasing amounts of CYB5A expression plasmid with expression plasmids for hCYP2E1, CYP2E1, CYP2A19, CYP2C33v4, and CYP1A1. The transfected cells were subsequently incubated with chlorzoxazone at levels on the linear part of the kinetic curve; this was 300  $\mu$ M CLZ for hCYP2E1, porcine CYP2E1, and CYP2A19 and 100  $\mu$ M CLZ for CYP2C33 and CYP1A1. A significant stimulatory dose response for CYB5A was found for three P450s (Fig. 4).



FIG. 1. Example of a typical Western blot showing expressed V5-His tagged porcine P450s and hCYP2E1 as visualized by staining with anti-V5 antibody. All P450s are coexpressed with POR, CYB5R3, and CYB5A (data not shown); each well contains 20  $\mu$ g of protein.

A 73, 85, and >300% increase in CLZ activity was seen with CYP2C33v4, CYP2E1, and hCYP2E1 at the highest level of CYB5A transfected, compared with the control without CYB5A. There was no significant response observed by cotransfection of CYB5A with CYP2A19 or CYP1A1 (data not shown).

### Discussion

The present study investigated the specificity of CLZ as a probe substrate for porcine CYP2E1 and the effects of CYB5A on CLZ catalytic activity. We show that the metabolism of CLZ is not limited to CYP2E1 in the pig and that CYP1A1, CYP2A19, and CYP2C33v4 also metabolize CLZ. We investigated hCYP2E1 along with porcine CYP2E1 to compare kinetic parameters for these homologs within the same system. The apparent  $K_{\rm m}$  for porcine CYP2E1 was more than double that of hCYP2E1, and the  $V_{\rm max}$  of hCYP2E1 was almost double that of porcine CYP2E1. Anzenbacherová et al. (2005) found similar results in microsomal systems, with CLZ hydroxylation in human greater than in conventional pig. Baranová et al. (2005) also found that CYP2E1 from minipig exhibited lower affinity for the substrate in both reconstituted and microsomal systems with  $K_{\rm m}$ values being  $\sim$ 1.2- to 4.7-fold greater compared with those for the human counterpart. CYP2E1 from the minipig and conventional pig is comparable with sequences differing by a single amino acid: a valine at position 346 is found in place of an aspartic acid in the conventional pig (Baranová et al., 2005). Other studies have also concluded that the enzymatic parameters for CLZ metabolism differ among species. Bogaards et al. (2000) observed a range of kinetic parameters in mouse, rat, rabbit, dog, minipig, monkey, and man with  $K_{\rm m}$  values ranging from 25 to 306  $\mu$ M and  $V_{\rm max}$  values ranging from 285 to 3184 pmol/min/mg microsomal protein. Although the magnitudes of the apparent kinetic parameters are unique to the system in which they are studied, the overall conclusion that the porcine CYP2E1 is capable of CLZ metabolism, although it is less efficient than hCYP2E1 can be drawn.

Evidence supporting the contribution of other porcine P450 enzymes to CLZ metabolism has also been reported in literature. Court et al. (1997) described a two-enzyme model for the metabolism of CLZ in porcine hepatic microsomes, which suggests the involvement of two kinetically distinct enzymes with different affinities for the same substrate (Madan et al., 2002). Skaanild and Friis (2007) argue that CLZ is a poor probe substrate for porcine CYP2E1; after treatment of porcine microsomes with anti-human CYP2A6 antibody, CLZ activity was reduced by an average of 65%, suggesting that porcine CYP2A may contribute equally to metabolism. Myers et al. (2001) treated barrows with inducers specific for the human CYP1A, CYP2B, and CYP3A subfamilies, which increased apparent levels of porcine CYP1A, CYP2B, and CYP3A proteins with no apparent effect on CYP2A or CYP2E1 levels; however, CLZ activity was increased in these treated liver fractions. This result suggests that porcine CYP1A, CYP2B, and CYP3A may also contribute to CLZ hydroxylation. Our study demonstrates that porcine CYP2A19 and CYP1A1, but not CYP3A, are active with this substrate. However, it is possible that other isoforms of CYP3A, which may have CLZ hydroxylation activity, exist in the pig.

Antibodies and inducers may not have the same specificity for P450 isoforms across different species, and the sources of antibodies used in experiments may yield various results. The use of individual porcine cDNA-expressed enzymes allows for a more definitive characterization of the individual enzymes, showing which porcine P450 isoforms are capable of CLZ metabolism. Although we did not quantify the absolute amounts of P450s in our expression system, the expression level of each P450 was normalized based on the density of



FIG. 2. The production of 6-OH-CLZ by P450 isoforms that displayed sigmoidal kinetics and modeled by the Hill equation. A, hCYP2E1; B, porcine CYP2E1; C, CYP1A1. The corresponding Eadie-Hofstee plots displaying a hook shape, characteristic of sigmoidal data, are depicted as insets. Each point represents a mean of four experiments performed in triplicate (n = 12)  $\pm$  S.D.

the Western blots that were immunostained for the V5 epitope on each of the enzymes, and this normalization process accounted for small differences in the levels of expression of the different P450s.

Several human P450s other than hCYP2E1 have been shown to metabolize CLZ. Masimirembwa et al. (1999) assessed the specificities of human P450 substrates using microsomal yeast extracts containing cDNA-expressed enzymes. CLZ metabolism occurred at a high turnover with CYP1A1 in addition to CYP1A2 and CYP2E1. Carriere et al. (1993) observed an increase in CLZ metabolism in human hepatocytes after induction of CYP1A with 3-methylcholanthrene, in addition to microsomal yeast cells engineered to express CYP1A1. Carriere et al. (1993) also reported detectable CLZ hydroxylation activity by human CYP2C9 and CYP3A4 expressed in yeast microsomes; however, the turnover numbers reported were  $\sim$ 40-fold lower than that for CYP1A1. Yamazaki et al. (1995) demonstrated low levels of CLZ hydroxylation by human CYP3A4 in an *Escherichia coli* expression system. In contrast, Gorski et al. (1997) observed a reduction in metabolism of CLZ after treatment of human microsomes with CYP3A4 inhibitors and antibodies but observed a subsequent restoration of activity with the addition of  $\alpha$ -naphthoflavone, a CYP3A4 inducer. CLZ metabolism was also observed with human CYP2A6 and CYP1A1 expressed in microsomes from Blymphoblastoid cells. However, treatment of human microsomes with inhibitors specific for each P450 isoform did not show significant inhibition of CLZ activity. Therefore, it was concluded that CYP3A4 may make a significant contribution to CLZ metabolism in vivo,



FIG. 3. The production of 6-OH-CLZ by P450 isoforms that were modeled by the Michaelis-Menten equation. A, CYP2C33v4; B, CYP2A19. The corresponding Eadie-Hofstee plots displaying a linear shape are depicted as insets. Each point represents a mean of four experiments performed in triplicate  $(n = 12) \pm S.D.$ 

#### TABLE 2

#### Summary of kinetic parameters for CLZ hydroxylation by porcine P450s and hCYP2E1

Kinetic parameters for hCYP2E1 porcine CYP2E1 and porcine CYP1A1 were modeled by the Hill equation, because these isoforms displayed sigmoidal kinetics. Kinetic parameters for porcine CYP2C33v4 and CYP2A19 were modeled by the Michaelis-Menten equation.

P450	K <sub>m</sub>	$V_{\rm max}$	$C_{\rm int}$
	$\mu M$	pmol/h/mg total cell protein	
hCYP2E1	$131.8 \pm 11.4$	$8650 \pm 356$	65.6
CYP2E1	$290.3 \pm 17.4$	$4980 \pm 232$	17.1
CYP2A19	$212.1 \pm 39.1$	$6680 \pm 441$	31.5
CYP2C33	$126.3 \pm 17.9$	$2100 \pm 93.3$	16.6
CYP1A1	$159.5\pm10.0$	$1650 \pm 55.3$	10.3

whereas CYP2A6 and CYP1A1 probably do not. We did not find detectable levels of 6-OH-CLZ produced with porcine CYP3A; this may be an example of a difference in metabolic profiles among species.

The effect of CYB5A on the catalytic properties of porcine P450 enzymes has not been investigated extensively. The ability of porcine CYB5A to modify the activity of CYP17A1 in the pig has been reported (Billen and Squires, 2009). The contribution of CYB5A to P450-mediated catalysis is complex and is dependent on the substrate and P450 isoform (Schenkman and Jansson, 2003). In some cases, CYB5A can modify the reaction by having an inhibitory or stimulatory effect and in other cases the effect has been shown to be obligatory. Our results show that the effects of CYB5A on CLZ



FIG. 4. The significant effect on CLZ hydroxylation by cotransfection with increasing amounts of CYB5A expression plasmid along with expression vectors for CYP2C33v4 (A), porcine CYP2E1 (B), and hCYP2E1 (C). There was a significant increase in the CLZ hydroxylation rate by increasing the levels of CYB5A transfection by linear regression analysis (P < 0.001). Data are presented as a mean of four experiments performed in triplicate (n = 12)  $\pm$  S.D.

hydroxylation activity are isoform-specific, because it only significantly augmented activity of porcine CYP2E1 and CYP2C33v4 and human CYP2E1. In a study by Yamazaki et al. (1996), the presence of CYB5A was shown to enhance the catalytic activity of hCYP2E1 toward CLZ, with only 7% activity reported in the absence of CYB5A. Gillam et al. (1994) also found that metabolism of CLZ was higher in the presence of human CYB5A with a decrease in  $K_{\rm m}$  and increase in  $V_{\text{max}}$ . Although the exact mechanism of CYB5A in P450 catalysis is unknown, studies using apo-CYB5A, which is devoid of heme, show no enhancement of activity toward CLZ by hCYP2E1, suggesting that the contribution of CYB5A is dependent on its electron-transferring properties (Yamazaki et al., 2002). CYB5A has been shown to reduce the competition for POR, at least between human CYP2A6 and CYP2E1, enhancing the activity of CYP2E1, which suggests that CYB5A enhances electron transfer (Tan et al. (1997). Studies have reported a lack of effect by CYB5A on the activity of human CYP1A1/2 toward ethoxyresorufin and phenacetin (Yamazaki et al. 2002), whereas some have observed a slight augmenting effect with 2-aminoanthracene (Duarte et al., 2007). Yamazaki et al. (2002) also reported an augmenting effect of CYB5A on CYP2A6-catalyzed coumarin and nicotine metabolism. We did not find an effect of CYB5A on CLZ metabolism with either CYP2A19 or CYP1A1. The contribution of the human CYP2C subfamily to CLZ metabolism has not been reported; Gorski et al. (1997) did not detect 6-OH-CLZ in B-lymphoblastoid microsomes expressing human CYP2C9. With the

two porcine CYP2C isoforms investigated, only CYP2C33v4 was efficient in metabolizing CLZ and CYB5A increased this activity.

The tissue content of various P450 isoforms and their activities determines their contribution to the metabolism of a substrate in vivo (Bertz and Granneman, 1997; Rendic and Di Carlo, 1997). The contribution of enzymes to reactions is determined by the  $V_{\text{max}}/K_{\text{m}}$ ratio in addition to the levels of their expression. Our results indicate that CYP2E1, CYP1A1, and CYP2C33v4 have a lower  $V_{\text{max}}/K_{\text{m}}$  ratio than CYP2A19. This result suggests that CYP2A19 is mostly responsible for CLZ hydroxylation; however, the relative abundance of these isoforms in vivo must be considered. Currently, information about the abundance of various porcine P450 isoforms is limited. Kojima and Morozumi (2004) found that hepatic expression of CYP2C33v4 and CYP2E1 mRNA was greatest, followed by CYP2C49 > CYP1A1 and CYP2A19 > CYP2B22. Therefore, it is possible that CYP2C33v4 and CYP2E1 maybe be the main isoforms responsible for CLZ metabolism. However, Szotáková et al. (2004) found no correlation between CLZ metabolism and CYP2E1 protein from immunoblotting. Thus, conclusions about the contribution of porcine P450s to CLZ metabolism in vivo are highly depend on liver abundance, which is yet to be conclusively determined.

In summary, we present definitive evidence for the contribution of four porcine P450s to CLZ metabolism, with novel information about the contribution of CYB5A in this metabolism for some isoforms. The porcine enzymes are similar to their human homologs in terms of CLZ metabolism and CYB5A augmentation. As in humans, CYP1A1, CYP2E1, and CYP2A19 are capable of CLZ hydroxylation in pigs. However, species differences are also evident because the porcine CYP3A does not metabolize CLZ. We have shown that CYP2C33v4 contributes to CLZ hydroxylation, although human CYP2C9 does not appear to metabolize CLZ; it is not clear whether other human CYP2C isoforms do. A better understanding of porcine P450 isoform abundance is needed before conclusions can be drawn about the use of CLZ as an in vivo probe for porcine CYP2E1.

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