ORIGINAL ARTICLE

Porcine CYP2A19, CYP2E1 and CYP1A2 forms are responsible for skatole biotransformation in the reconstituted system

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Abstract

OBJECTIVES: To study the contribution of individual purified porcine CYP1A2, 2E1 and 2A19 enzymes to the biotransformation of skatole.
METHODS: Individual porcine and human enzymes (CYP1A2, 2E1 or 2A6/19) were used to study their potential involvement in skatole metabolism. Furthermore, the inhibition experiments using specific inhibitors of CYP1A2, 2E1 or 2A6/19, were performed. For determination of skatole biotransformation by individual CYP forms in reconstituted systems, HPLC method with UV detection was used.
RESULTS: The data presented in this paper show that porcine and human CYPs are responsible for the formation of indole-3-carbinol and 3-methyloxindole. Whereas in pig CYP2A19 and CYP1A2 seem to be the most important for metabolism of skatole, in man CYP1A2 and CYP2E1 forms are mainly responsible for the production of the metabolites mentioned above.
CONCLUSIONS: The porcine and human CYP1A2, 2E1, 2A6/19 forms contribute to formation of 3-methyloxindole and indole-3-carbinol.

INTRODUCTION

Skatole (3-methylindole, abbrev. 3MI) is a naturally occurring metabolite produced from tryptophan in gastrointestinal tracts of pigs, humans and other species (Dehnhard *et al.* 1991). Skatole has been established as a pneumotoxin in several species. Bioactivation of skatole and covalent binding were shown to result in toxicity mediated by cytochrome P450 enzymes (CYP); however, toxic implications of the exposure in man have not been fully assessed yet. Humans can be exposed to this compound through intestinal absorption and by cigarette smoke (Lanza & Yost, 2001). However, pigs, contrary to other species, are not susceptible to 3MI toxicity, suggesting that pigs may metabolize this compound by different metabolic pathways (Diaz *et al.* 1999; Thornton-Manning *et al.* 1996). On the other hand, the most important implication of the presence of 3MI in a tissue is its presence in pork meat. Uncastrated (intact) male pigs are used for meat production in several countries due to better feed conversion and better composition of fatty acids compared with castrated pigs. However, some intact male pigs carry so-called boar taint, an undesirable off-odour released when meat is heated. Boar taint is caused by the presence of 3MI and/or androstenone in fat.

Abbreviations:
P450 (CYP) – Cytochrome P450
3MI – 3-methylindole
3MOI – 3-methyloxindole
I3C – indole-3-carbinol

It is not known why only a certain percentage of a given population accumulates 3MI in fat to a level that can be detected by humans. Several studies have been performed to elucidate this fact. Nutrition is important in the regulation of 3MI intestinal production (Claus *et al.* 2003; Zamaratskaia *et al.* 2005). Furthermore, metabolic processes in the liver play an essential role in 3MI accumulation (Zamaratskaia & Squires, 2009). In pigs, the role of CYP2A19 (orthlogous form to human CYP2A6) and CYP2E1 is considered (Doran *et al.* 2002; Terner *et al.* 2006). It is also known that skatole metabolites do not contribute to the boar taint (Diaz & Squires, 2000a). Until now, the approach used in studies on identification of the CYP forms involved in skatole metabolism has been indirect, relying on the use of inhibitors of CYP activities in microsomal fractions. On the other hand, it has also been shown that the inhibitors used are less specific when used in experiments with pig microsomes or whole hepatocytes (Diaz & Squires, 2000a; Terner et al. 2006) which only stresses the need to confirm the role of individual CYP forms in skatole metabolism by direct methods i.e. by using enzyme systems containing only one form of CYP. Several human CYPs contributing to 3MI biotransformation have been identified (Lanza & Yost, 2001), using recombinant CYPs. However, similar study has not been performed in pigs yet, because porcine CYPs are not commercially available. The use of purified enzyme isolated as described in this paper can help not only in the identification of the particular form involved in skatole biotransformation, but also in further studies on skatole metabolism. Detail information about differences or similarities in drug metabolism in humans and other species is important in the field of experimental pharmacology, because it should provide important information for the prediction of metabolism of drugs and other xenobiotics (Krizkova *et al.* 2008; Matal *et al.* 2008). In particular, the pathways of drug and xenobiotic metabolism in pig are studied as the pig can serve as an experimental animal in pharmacological and toxicological studies; moreover, pig hepatocytes are intended for xenotherapy or for construction of bioartificial liver supporting devices (Couzin, 2002; Desille *et al.* 1999).

Skatole metabolism in pigs should be studied because of the current need to find possible ways to eliminate boar taint without surgical castration e.g. stimulation of skatole metabolism as the skatole metabolites are known not to exhibit unwanted properties of the parent compound (Diaz & Squires, 2000a). The objective of this study was to elucidate the contribution of porcine CYP1A2, CYP2E1 and CYP2A19 to skatole hepatic metabolism.

MATERIAL AND METHODS

Material

All chemicals were purchased from Sigma-Aldrich (Prague, Czech Republic), if not stated otherwise and were in purity of best available. Pooled pig liver microsomes (8 pigs) were prepared according to established methods (Philips & Shephard, 1998) from experimental material obtained in a local slaughterhouse. Authentic standard 3-hydroxy-3-methyloxindole was a generous gift provided by Dr. G. S. Yost, Department of Pharmacology and Toxicology, University of Utah (Salt Lake City, UT). Porcine CYP2E1 enzyme was kindly donated by Dr. Jana Baranová and Dr. Eva Anzenbacherová (Baranová *et al.* 2005), purified pig CYP1A2 and CYP2A19 forms were isolated by procedure described by Matal *et al.* (2009). Shortly, the solubilized porcine liver microsomes were applied on octylamino-Sepharose column and fractions rich on desired CYP were applied on hydroxylapatite column. After repeated hydroxylapatite chromatography, the pure forms were obtained. Human NADPH:cytochrome P450 oxidoreductase (P450 reductase), human CYP2E1 and human CYP1A2 were purchased from Panvera (Panvera-Invitrogen, Carlsbad, CA). Human CYP2A6 containing E. coli membranes was kindly donated by Dr. P. Souček (National Institute of Public Health, Prague, CZ), the human enzyme was then prepared by the method described earlier (Soucek 1999). Pig P450 reductase was isolated by affinity chromatography (Yasukochi & Masters, 1976), pig cytochrome b_5 was isolated by method described in Soucek *et al.* (2001). For analysis of 3MI and its metabolites, HPLC system (Shimadzu LC-20A Prominence, Kyoto, Japan) with UV detection was used.

Methods

Enzyme assays. Skatole incubations were performed in the reconstituted system containing 25 pmol of CYP1A2 (or 2E1, 2A6) enzyme (human or porcine), 75 pmol of P450 reductase, and 100 pmol of cytochrome b_5 (1:3:4). After 10 minutes at room temperature, 10 pmol of sonicated L-α-dilauroylphosphatidylcholine, which had been preincubated for 20 min at room temperature, was added. This mixture was incubated with 0.66 mM 3MI, 1 mM EDTA in 50 mM phosphate buffer pH 7.4. After 5 minutes, the NADPH generating system (consisting of $NADP^+$ 0.8 mM, sodium isocitrate 5.8mM, isocitrate dehydrogenase 0.3 units/ml and $MgSO_4$ 8 mM) was added to a final volume of 200 µl. The reaction mixture was incubated at 37°C for 90 min and stopped by addition of 200 µl of ice-cold acetonitrile. The mixture was then centrifuged at 3000 g for 20 min and 50-µl aliquot of the supernatant was analyzed by HPLC-UV. Production of metabolites was found to be linear over a range of 60 to 120 min. For negative control incubations, the acetonitrile was added prior to incubating. All reactions were performed in duplicates. For the detection of

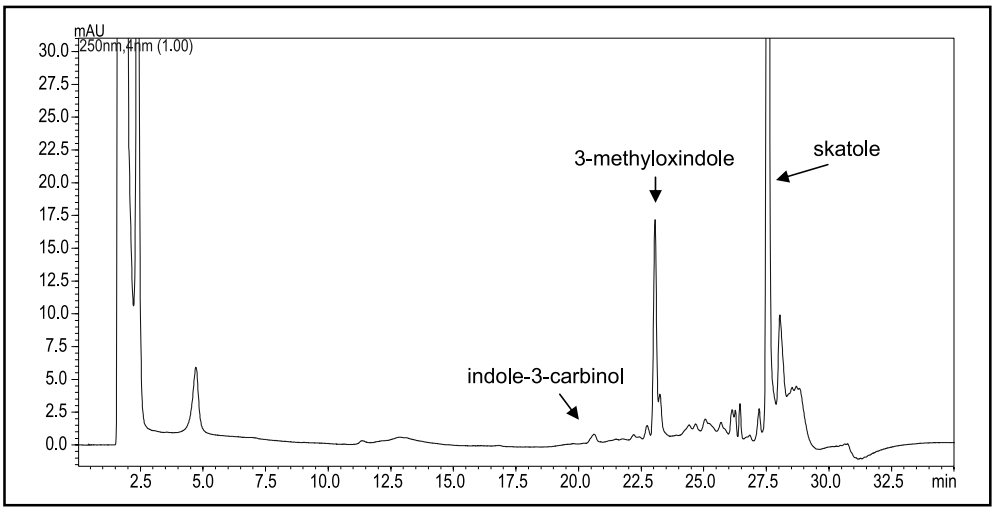

Fig. 1. Chromatogram of skatole incubation in the reconstituted system with porcine CYP1A2 enzyme.

skatole metabolites, the method described in original paper of Diaz *et al.* (1999) was used. HPLC analysis was conducted immediately after the incubations. Metabolites were identified by comparison of retention times and spiking the metabolite mixture with authentic standards.

Inhibition experiments. The inhibition experiments were performed with isolated CYPs to verify the formation of 3MI metabolites. For CYP1A2 the furafylline (Kunze & Trager, 1993; Zamaratskaia & Zlabek, 2009), for CYP2E1 the diethyldithiocarbamate (Baranová *et al.* 2005) and for CYP2A19 (CYP2A6) the 8-methoxypsoralen (Koenigs *et al.* 1997; Li *et al.* 2006), was used. The concentration of inhibitors used was as follows: 50 μM furafylline for CYP1A2, 25 μM 8-methoxypsoralen for CYP2A19 and 30 μM diethyldithiocarbamate for CYP2E1, for details see Matal *et al.* (2009) and Baranova *et al.* (2005).

RESULTS

Individual human and porcine CYP forms were incubated with 3MI in reconstituted system to determine their involvement in formation of 3MI metabolites. In ***Fig. 1***, the typical HPLC chromatogram is shown to document the formation and separation of the products using individual CYPs.

3-methyloxindole seems to be a predominant metabolite formed by used porcine CYPs, indole-3-carbinol is of a lower abundance. In accordance with the literature, similar results were obtained using human recombinant CYPs (results not shown, for detailed information see Thornton-Manning *et al.* 1996; Lanza & Yost, 2001). No other metabolites formed by porcine CYPs were detected. The CYP activities, calculated as nanomoles of 3MOI and I3C produced per minute per nmol of P450 are shown in ***Table 1***. In man, CYP1A2 and CYP2E1 were the most potent, whereas in pig, CYP2A19 together with CYP1A2 were the most active in formation of 3MI metabolites.

For the confirmation of formation of 3MI metabolites by respective CYPs, the inhibition experiments with specific inhibitors of individual CYP activities were performed in all cases. At least 70% decrease in amounts of metabolites formed was observed in each assay confirming the specificity of an involvement of a particular CYP form (either human or porcine) in the respective reaction.

Table 1. Rate of production of 3MI metabolites (3MOI – 3-methyloxindole; I3C – indole-3-carbinol) by individual human and pig CYPs in the reconstituted system; N.D., not detected.

	pig		man	
	3MOI (nmol of product/ nmol P450/min)	I3C (nmol of product/ nmol P450/min)	3MOI (nmol of product/ nmol P450/min)	I3C (nmol of product/ nmol P450/min)
CYP1A2	1.65	0.32	3.65	2.72
CYP2E1	1.47	N.D.	5.15	0.12
CYP2A19/2A6	2.99	0.44	0.1	N.D.

Table 2. Inhibitory effect of 50 μM furafylline (CYP1A2), 25 μM 8-methoxypsoralen (CYP2A6/2A19) and 30 μM diethyldithiocarbamate (CYP2E1) on amounts of 3MOI and IC3 formed in the reaction mixture with respective inhibitor. Values are shown as percent of control activity.

	pig		man	
	3MOI	I3C	3MOI	I3C
CYP1A2	18.1	11.2	5.3	0.7
CYP2E1	29.5	-	20.6	25.4
CYP2A19/2A6	0.2	0.9	29.1	-

DISCUSSION

3MI is a non-polar substance formed by intestinal bacteria in the hindgut of several species including man and pig. No physiological function of skatole is known. Its major undesirable effect is a fecal-like odour of the meat of intact male pigs as this compound accumulates in the fat in concentration high enough to cause a boar taint. In the past few years, several studies were done to determine individual CYPs responsible for 3MI metabolism, using specific CYP inhibitors. It is also known that 3MI level in fat negatively correlates with activities of CYP2E1 and CYP2A19 (Friis *et al.* 1995; Diaz & Squires, 2000a; Zamaratskaia *et al.* 2005). According to these findings, CYP2E1 and CYP2A19 seemed to be the most important. However, to determine conclusively the participation of an individual CYP in the formation of 3MI metabolites, the use of pure enzyme is needed. In our experiments, isolated pure porcine CYP1A2, 2A19 and CYP2E1 obtained previously (Baranova *et al.* 2005; Matal *et al.* 2009) were used as well as their human orthologous forms for comparison.

Our results show that porcine CYP1A2, CYP2E1 and CYP2A19 are able to form two 3MI metabolites, namely 3MOI and I3C. According to the results from previous experiments, 3MOI is one of the major metabolites formed (Diaz et al, 1999). In the pig, the CYP2A19 seems to be of major importance, as this enzyme is able to form the metabolites in the highest rate (c.f. Tab. 1). The importance of CYP2A19 in 3MI metabolism was also found by Diaz & Squires (2000a) in the microsomes, using inhibitors of CYP activities. In the man, the CYP2A6 is probably the less important enzyme in this respect according to the comparison with the other CYP forms used in this experiment. However, the polymorphism in *CYP2A6* gene was detected in the man, resulting in altered substrate specifity (Nunoya *et al.* 1998). Interestingly, in the work of Diaz & Squires (2000a), the 196-fold difference in coumarin 7-hydroxylase activity and 607-fold difference in CYP2A19 content were detected among pig samples. This extremely high variability in pigs can be a result of the genetic polymorphism resulting in great differences in 3MI metabolism leading to the boar taint and hence explaining differences between pigs in exhibiting the boar taint. Additionally, the activities of CYP2E1 and CYP2A are affected by the presence of testicular steroids (Doran *et al.* 2002; Gillberg *et al.* 2006; Zamaratskaia *et al.* 2009).

The porcine CYP1A2, as for the first time described in this study, is able to metabolize skatole to 3MOI and I3C, although until now there was no evidence for the role of porcine CYP1A2 in skatole metabolism. However, in the work of Terner *et al.* (2006), the formation of 3MOI and 3-hydroxy-3-methyloxindole in primary cultured pig hepatocytes was not completely affected by CYP2E1 and CYP2A19 inhibitors, indicating that also other enzymes may play a role in this process. As it is known that formation of 3-hydroxy-3-methyloxindole is catalyzed by the aldehyde oxidase, which is a cytosolic enzyme (Diaz & Squires, 2000b), also other enzymes were supposed to be implicated. Formation of I3C and 3MOI was also detected in experiments with human recombinant CYP1A2 (Lanza & Yost, 2001). In the work of Messina *et al.* (2008), enzymatic properties and substrate specificity of porcine CYP1A2 were shown to be similar to those of the corresponding human enzyme. Taken together, the porcine CYP1A2 is, according to presented results, responsible for 3MI biotransformation in pig, which has never been published before.

The CYP2E1 was found to be involved only in 3MOI formation in pig, whereas in man, it was able to form also the I3C metabolite. In pig, the I3C is a minor metabolite of skatole, contributing to its metabolism by 2.7% (Diaz *et al.* 1999). In the study of Diaz *et al.* (1999), seven metabolites formed *in vitro* by pig liver microsomes were identified, but only three in primary cultured pig hepatocytes (Terner *et al.* 2006) were found. As mentioned above, also the cytosolic enzymes (namely the aldehyde oxidase) play a role in *in vitro* metabolism of skatole (Diaz & Squires, 2000b). As in this work two skatole metabolites were detected, possible involvement of other enzymes involved in formation of the other skatole metabolites in pig should be further studied. In conclusion, liver microsomal CYP2A19, CYP2E1 and CYP1A2 are involved in the metabolism of 3MI in pigs, particularly in formation of 3MOI (CYP2A19, CYP2E1 and CYP1A2) and I3C (CYP2A19 and CYP1A2).

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