Regulation of CYP2A6 Protein Expression by Skatole, Indole, and Testicular Steroids in Primary Cultured Pig Hepatocytes

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ABSTRACT:
CYP2A6 is one of the enzymes involved in the hepatic metabolism of a naturally produced compound, skatole, in the pig. Low CYP2A6 activity has been linked to excessive accumulation of skatole in pig adipose tissue and development of the phenomenon “boar taint.” CYP2A6 activity varies between male and female animals, suggesting the involvement of sex hormones in regulation of the enzyme activity and/or expression. The present study investigated whether pig hepatic CYP2A6 protein expression is regulated by the testicular steroids testosterone, androstenone, or estrone sulfate using primary cultured hepatocytes as a model system. The study has also examined whether CYP2A6 expression can be modulated by the boar taint compounds skatole and indole. The research has established that androstenone inhibits CYP2A6 protein expression at the concentration of 1, 10, and 100 nM by 55, 37, and 44%, respectively. In contrast to androstenone, skatole and indole (final concentrations, 1, 10, and 100 nM) had a stimulatory effect on CYP2A6 expression. The effect of indole was more pronounced than that of skatole (maximum induction by 145 and 70%, respectively). Estrone sulfate and testosterone did not have a significant effect on CYP2A6 protein level. This is, as far as we know, the first communication to report the regulation of pig hepatic CYP2A6 expression by steroids and boar taint compounds. The hormonal modulation of CYP2A6 expression might contribute to gender-related differences in pig hepatic CYP2A6 activity and skatole accumulation in pig adipose tissue.

CYP2A6 is one of the microsomal enzymes involved in the metabolism of xenobiotics in humans and a number of other species (Pearce et al., 1992; Endo et al., 2007). Although CYP2A6 represents only 4% of human hepatic P450 (Shimada et al., 1994; Guengerich, 1995), it plays an important role in the metabolism of nicotine, coumarin, and a number of pharmaceutical agents (Miles et al., 1990; Nakajima et al., 2006). In pigs, hepatic CYP2A6 (or CYP2A19, according to the pig nomenclature), alongside CYP2E1, is involved in degradation of a naturally occurring compound, skatole (3-methylindole) (Squires and Lundström, 1997; Diaz and Squires, 2000). Skatole is produced by bacterial transformation of L-tryptophan in the pig intestine (Yokoyama and Carlson, 1979). Because of its lipophilic properties, skatole can be accumulated in pig adipose tissue, where it contributes to the phenomenon of boar taint, an unpleasant odor of some cooked pork (reviewed by Bonneau, 1982). One of the reasons for high skatole accumulation in adipose tissue is a low rate of skatole degradation in pig liver. CYP2E1 is thought to have a major input in the metabolism of skatole in pigs. CYP2E1 activity, protein expression, and mRNA are low in pigs with high adipose tissue skatole content (Babol et al., 1998; Doran et al., 2002b). CYP2A6 activity also correlates negatively with skatole level (Diaz and Squires, 2000), although the input of CYP2A6 in skatole metabolism is thought to be less significant than that of CYP2E1 (Terner et al., 2006).

The mechanisms regulating CYP2E1 activity and expression in pig liver have been extensively studied, although only limited information is available on the regulation of pig hepatic CYP2A6 (Terner et al., 2006; Zamaratskaia et al., 2007). Lin et al. (2004) demonstrated that low CYP2A6 activity in some pigs is related to a functional polymorphism in the coding region of the corresponding gene and suggested that this polymorphism contributes to high skatole accumulation. However, Skinner et al. (2006) found no relation between this CYP2A6 polymorphism and skatole level in a study on a large population of commercial Danish pigs. Moreover, Skaanild and Friis (2005) have suggested that differences in pig hepatic CYP2A6 activity in minipigs are due not to polymorphism but to transcriptional regulation of the enzyme expression. The mechanisms regulating the expression of pig hepatic CYP2A6 remain unknown.

It has been established that CYP2A6 activity varies between male and female pigs and between castrated and intact pigs (Skaanild and Friis, 1999; Zamaratskaia et al., 2006), which suggests the involvement of sex steroids in regulation of this enzyme. Sex-related differences in activities of the CYP2A enzymes have been previously reported for hamsters (Pelkonen et al., 1994). Studies on the hormonal...
regulation of the pig hepatic CYP2A6 are limited to the communication by Zamarskaia et al. (2007), who demonstrated that androstenone and 17β-estradiol modulate CYP2A6 activity in isolated microsomes. It is unknown whether any other sex steroids affect the porcine CYP2A6 activity or expression.

Enzyme expression can be regulated by the level of its substrates. Previous experiments with primary cultured pig hepatocytes have established that expression of CYP2E1 protein can be up-regulated by at least one of the substrates, namely skatole (Doran et al., 2002a). Whether skatole or other boar taint compounds can affect expression of the skatole-metabolizing enzyme CYP2A6 is unknown. The aim of the present study was to investigate effects of the testicular steroids (androstenone, testosterone, and estrone sulfate) and nonsteroid boar taint compounds (skatole and indole) on the expression of CYP2A6 protein using primary cultured pig hepatocytes as a model system.

Materials and Methods

**Chemicals.** Skatole (3-methylindole), indole (2,3-benzopyrole), androstenone (5α-androst-16-en-3-one), testosterone (17β-hydroxy-4-androsten-3-one), estrone 3-sulfate sodium salt [1,3,5(10)-estratrien-17-one 3-sulfate], inhibitors of proteolytic enzymes (antipain, pepstatin, leupeptin), collagenase type IV, HEPES-buffered Hanks’ balanced salt solution, and phosphate-buffered saline (PBS) were from Sigma (Dorset, UK). Collagen-coated plates (100-mm diameter) were provided by Appleton Woods (Birmingham, UK). A polyclonal rabbit antibody against human cytochrome P450 2A6 was from QED Bioscience Inc. (San Diego, CA). Horseradish peroxidase-linked donkey anti-rabbit IgG and an enhanced chemiluminescence detection system were from GE Healthcare (Buckinghamshire, UK). A nitrocellulose membrane for Western blotting (pore size, 0.45 μm) was from Bio-Rad Laboratories (Herts, UK). All other chemicals and reagents were purchased from Sigma. ImageQuant program was from GE Healthcare (Buckinghamshire, UK).

**Animals.** Entire male pigs of a commercial Large White crossbreed (40% Large White × 40% Landrace × 20% Duroc) from the same herd were used in the study. Animals were reared on a commercial standard pelleted diet (ABN, Peterborough, UK) and slaughtered in the European Union-approved abattoir of the Department of Clinical Veterinary Science, University of Bristol, in compliance with regulations for humane care and slaughter. Samples of liver from the left lateral lobe were collected within 5 min after slaughter and were stored at −80 °C until further analysis.

**Hepatocyte Isolation and Culture.** Hepatocytes were isolated as described in Doran et al. (2002a). Cell viability was determined by trypan blue exclusion and was approximately 90%. The isolated hepatocytes were plated on collagen-coated plates (approximately 6 × 10⁶ cells per plate) with 10 ml of Medium 199. The cells were preincubated for 24 h at 37°C in a humidified atmosphere or air (95%) and CO₂ (5%) to ensure attachment of the hepatocytes to the plates. After 24 h, the medium was replaced with fresh medium with or without treatment (experimental and control plates, respectively), and the cells were further incubated for 24 h under the same conditions. At 24 h after the treatment, the hepatocytes were washed twice with PBS, scraped into 0.3 ml of PBS with added inhibitors of proteolytic enzymes antipain + pepstatin + leupeptin (1 μg/ml), and snap-frozen in liquid nitrogen. The frozen cells were stored at −80°C until further analysis.

**Treatments.** Stock solutions of testosterone were prepared in ethanol. Stock solutions of androstenone, estrone sulfate, skatole, and indole were prepared in methanol. Two concentrations of stock solutions (10 μM and 0.2 mM) were used for each of the treatments to ensure that the maximum final concentration of methanol or ethanol added to the cells was not higher than 0.5%. Addition of methanol or ethanol alone at the final concentrations up to 0.5% had no effect on CYP2A6 protein expression.

**Analyses of CYP2A6 Protein Expression.** CYP2A6 protein expression in the primary cultured hepatocytes was analyzed by Western blotting following

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**Results**

**Effect of Skatole and Indole on CYP2A6 Protein Expression.**

The results presented in Fig. 1 show that skatole at the final concentrations of 1, 10, and 100 nM induces CYP2A6 protein expression in cultured primary hepatocytes by 70, 33, and 63%, respectively, compared with the control values. There were no statistically significant differences between the CYP2A6 protein levels in the presence of 1, 10, and 100 nM skatole. Further increases in skatole concentration to 500 and 1000 nM were accompanied by decrease in CYP2A6 protein expression, which returned to the control values in the presence of 1000 nM skatole.

Similarly to skatole, incubating the hepatocytes with indole also resulted in an increase in CYP2A6 protein expression (Fig. 2). In the case of indole, activation of CYP2A6 expression was more pronounced compared with similar concentrations of skatole. Thus, expression of CYP2A6 protein in the presence of 1, 10, and 100 nM indole was 105, 131, and 145% higher compared with the CYP2A6 protein level in the control samples. Further increase of indole concentration to 500 and 1000 nM, similarly to skatole, resulted in a gradual decrease in CYP2A6 protein expression and its return to the control level.

**Effect of Testicular Steroids on CYP2A6 Protein Expression.**
The effect of the testicular steroids androstenone, testosterone, and estrone sulfate on CYP2A6 protein expression in primary cultured hepatocytes is presented in Figs. 3, 4, and 5. Incubation with androstenone at the final concentrations 1, 10, and 100 nM resulted in a significant inhibition of CYP2A6 expression by 55, 37, and 44%, respectively, compared with CYP2A6 protein expression in control hepatocytes. The level of CYP2A6 protein returned to control values (and even somewhat exceeded these values) when androstenone concentration was further increased to 500 and 1000 nM.

In contrast to androstenone, incubation with testosterone and estrone sulfate did not have inhibitory effects on CYP2A6 protein expression at any of the concentrations studied (Figs. 4 and 5). Moreover, there was tendency for an increase in CYP2A6 protein level in the presence of 1 to 100 nM estrone sulfate (Fig. 5) and 500 and 1000 nM testosterone (Fig. 4). However, this increase was not statistically significant.

**Discussion**

An excessive accumulation of skatole in pig adipose tissue leads to development of the phenomena “boar taint.” One of the factors regulating adipose tissue skatole level is the rate of skatole clearance in pig liver. The first stage of hepatic skatole metabolism involves the P450 system, in particular the isoforms CYP2E1 and CYP2A6 (Babol et al., 1998; Diaz and Squires, 2000). Low activity and expression of these P450s lead to a reduced rate of hepatic skatole metabolism followed by skatole accumulation in pig adipose tissue (Diaz and Squires, 2000; Doran et al., 2002b).

In other species, skatole metabolism might take place in tissues other than the liver. For example, in ruminants, skatole is intensively metabolized in the lungs, and this process is accompanied by the formation of pneumotoxic intermediates leading to the development of pulmonary edema and emphysema (Yost, 1989; Ramakanth et al., 1994). The formation of pneumotoxic intermediates does not involve CYP2E1 or CYP2A6 and is mainly catalyzed by P450s of the 2F and 2B subfamilies (Ramakanth et al., 1994; Carr et al., 2003).

There is no information on whether skatole can be metabolized in extrahepatic tissues in pigs. However, the presence of the skatole-metabolising enzymes has been detected not only in the pig liver but also in a number of other tissues. Thus, Lin et al. (2004) and Lin et al. (2006) established that CYP2E1 mRNA is expressed in pig spleen, liver, muscle, ovary, kidney, and testes, whereas CYP2A6 mRNA was only present in liver and kidney. The expression of both CYP2E1 and CYP2A6 mRNA was much higher in liver compared with the other tissues.

The mechanisms regulating the expression of CYP450 isoforms in pig liver have not been completely understood. The present study has established that CYP2A6 protein expression in primary cultured hepatocytes can be induced by its substrates, skatole and indole. These results are consistent with the previous finding that skatole induces...
expression of the other skatole-metabolising enzyme, CYP2E1, in isolated pig hepatocytes (Doran at al., 2002a). In our previous experiments, we have investigated a time course of CYP2E1 induction by skatole, and we have reported that the maximum effect of skatole was observed between 20 and 28 h of the incubation (Doran et al., 2002a). Therefore, a 24-h incubation time was chosen for the present study. This incubation time is also consistent with the incubation periods used by other authors in the studies on P450 expression in isolated hepatocytes (Woodcroft and Novak, 1997; Wu et al., 1997).

In the present study, we have observed a biphasic response in CYP2A6 protein expression to skatole and indole treatments [i.e., an increase in the CYP2A6 level at low (physiological) concentrations of the treatments and a decline in the CYP2A6 protein level at high concentrations of the treatments]. The biphasic response of P450 enzymes to inducers is not uncommon. For example, Donato et al. (2000) reported an increase in CYP2A5 activity in cultured primary murine hepatocytes in the presence of low concentrations of porphyromogenic substance griseofulvin. This was followed by a decline in the CYP2A6 activity in the presence of high concentrations of griseofulvin. The reason for the biphasal response is not clear. One possible explanation could be a cytotoxic effect of the treatments at high concentrations.

An interesting observation in the present study is that the effect of indole on CYP2A6 protein expression was higher than the effect of skatole (on average 127 versus 55%). It is well known that enzyme expression can be induced by its substrates. We speculate that indole might be a preferable substrate for the porcine CYP2A6 and this might have input in the high induction of CYP2A6 protein expression by this compound. This hypothesis is consistent with observations of Gillam et al. (2000), who reported that CYP2A6 is the most active cytochrome in the formation of at least two products of oxidative indole metabolism and that CYP2E1 has less input in indole metabolism in this system than CYP2A6.

The degree of CYP2A6 induction by skatole (on average by 55%) was similar to that previously reported for the induction of CYP2E1 protein expression by skatole (by 45%) (Doran et al., 2002a). The mechanisms of CYP2A6 and CYP2E1 protein induction by skatole are unknown. According to the literature, the classic CYP2A6 inducers (i.e., phenobarbital, rifampicin, pyrazole) act at the mRNA level (Donato et al., 2000). Furthermore, Horn et al. (2002) have demonstrated that the oral administration of the indolic compound indole-3-carbinol to rats can up-regulate the mRNA expression for a number of hepatic P450s. Whether the induction of the porcine CYP2A6 and CYP2E1 protein expression by skatole and indole are related to an increase in their mRNA levels requires further investigation.

In respect to the testicular steroids, the present study has established that only androstenone (not testosterone or estrone sulfate) had a significant effect on CYP2A6 protein expression. The levels of steroid treatment used in this study are similar to steroid concentrations used by other authors for cell culture experiments (Schwenk and Lopez del Pino, 1980; Sinclair et al., 2005).

In the present work, low concentrations of androstenone have inhibited CYP2A6 expression. This is consistent with the previous observation that androstenone down-regulates the skatole-induced expression of CYP2E1 in cultured pig hepatocytes (Doran et al., 2002a). Whether this androstenone-mediated decrease in CYP2E1 expression will affect CYP2A6 activity is not clear. Some inhibition of CYP2A6 activity by androstenone was observed by Zamaratskaia et al. (2007). However, the effect of androstenone on CYP2A6 activity (Zamaratskaia et al., 2007) was smaller than the effects of androstenone on CYP2A6 protein expression (the present study). This might be partially explained by the use of different experimental systems (isolated microsomes and isolated hepatocytes, respectively) and different ranges of androstenone concentrations.

In the present study, the inhibitory effect of androstenone on CYP2A6 protein expression was only observed at low androstenone concentrations (1, 10, and 100 nM). The effect was abolished when androstenone concentrations were increased to 500 nM and 1000 nM. The mechanism of this biphasic response of CYP2A6 protein expression to androstenone treatment is unknown.

In contrast to androstenone, testosterone had no significant effect on CYP2A6 protein expression in our experiments. This finding is consistent with the results of Zamaratskaia et al. (2007), who demonstrated that testosterone does not alter CYP2A6 enzyme activity in hepatic microsomes isolated from entire male pigs.

The mechanism of the inhibitory effect of androstenone on CYP2A6 protein expression has not been defined yet. In the case of CYP2E1, it has been demonstrated that androstenone (but not testosterone) inhibits enzyme expression at the level of transcription via inhibition of binding of one of the transcription factors, COUP-TF1 (Tambyrajah et al., 2004). It is possible that the inhibitory effect of androstenone on CYP2A6 expression is also mediated via prevention of binding specific transcription factors to the CYP2A6 promoter.

We suggest that the previously reported sex and age differences in skatole level (Babol et al., 2004; Zamaratskaia et al., 2006) are related to an inhibitory effect of androstenone (and possibly some other sex steroids) on the expression of the skatole-metabolizing enzymes CYP2E1 and CYP2A6. The low CYP2E1 and CYP2A6 expression might lead to a reduced rate of the hepatic skatole clearance with the consequent accumulation of skatole in adipose tissue.

It has been previously reported that an increase in skatole level in pig adipose tissue is accompanied by an elevated level of estrone sulfate in plasma and fat (Babol et al., 1999; Zamaratskaia et al., 2005). Although the present study found no significant effect of estrone sulfate on the expression of CYP2A6 protein in primary cell hepatocytes, it does not exclude the possibility that estrone sulfate directly affects CYP2A6 activity without influencing CYP2A6 expression.

![Graph](image.png)
This is the first study to investigate the role of testicular steroids and nonsteroid boar taint compounds on the expression of pig hepatic CYP2A6. The main finding of this research was that CYP2A6 protein expression could be induced by physiological concentrations of skatole and indole and induced by androstenone in primary cultured hepatocytes. The results of this study have contributed to understanding the mechanisms regulating the expression of pig hepatic CYP2A6.

References


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