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Regulation of cytochrome P450 2A6 protein expression by skatole, indole and testicular steroids in primary cultured pig hepatocytes

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Abbreviations: CYP450, cytochrome P450; CYP2E1, cytochrome P450 2E1; CYP2A6, cytochrome P450 2A6; PBS, phosphate buffered saline; PBST, phosphate buffered saline tween 20; ECL, enhanced chemiluminescence detection system, SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Abstract

Cytochrome P4502A6 (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 males and females, 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 sulphate, 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 concentration 1, 10 and 100 nM) had a stimulatory effect on CYP2A6 expression. The effect of indole was more pronounced compared with the effect of skatole (maximum induction by 145% and 70% respectively). Estrone sulphate and testosterone did not have a significant effect on CYP2A6 protein level. This is, as far as we know, the first communication which reports 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.

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Cytochrome P450 2A6 (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, are involved in degradation of a naturally occurring compound, skatole (3-methylindole) (Squires and Lundstrom, 1997; Diaz and Squires, 2000). Skatole is produced by bacterial transformation of L-tryptophan in the pig intestine (Yokoyama and Carlson, 1979). Due to its lipophilic properties, skatole can be accumulated in pig adipose tissue, where it contributes to the phenomenon of boar taint, an unpleasant odour 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., 2002a). 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, whilst 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) did not find any relation between this CYP2A6 polymorphism and skatole level in a study on a large

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population of commercial Danish pigs. Moreover, Skaanild and Friis (2005) have suggested that differences in pig hepatic CYP2A6 activity in minipigs are not due to polymorphism but to transcriptional regulation of the enzyme expression. The mechanisms regulating the expression of pig hepatic CYP2A6 remains unknown.

It has been established that CYP2A6 activity varies between males and females 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 Zamaratskaia et al (2007) who demonstrated that androstenone and 17β-oestradiol 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., 2002b). Whether skatole or other boar taint compounds can affect expression of the skatole-metabolising enzyme CYP2A6 is unknown.

The aim of the present study was to investigate effects of the testicular steroids (androstenone, testosterone and estrone sulphate) and non-steroid boar taint compounds (skatole and indole) on the expression of CYP2A6 protein using primary cultured pig hepatocytes as a model system.

Methods

Chemicals. Skatole (3-methylindole), indole (2,3-benzopyrrole), androstenone (5α-androst-16-en-3-one), testosterone (17β-hydroxy-4-androsten-3-one), estrone 3-sulphate sodium salt (1,3,5(10)-estratrien-17-one 3-sulphate), inhibitors of proteolytic enzymes (antipain, pepstatin, leupeptine), collagenase type IV, HEPES-buffered Hanks' balanced salt solution and phosphate buffered saline (PBS) were from Sigma (Dorset, UK). Medium 199, foetal bovine serum, L-glutamine and penicillin/streptomycin were purchased from Gibco BLR (Paisley, UK). Collagen-coated plates (diameter 100 mm) were provided by Appleton Woods (Birmingham, UK). A polyclonal rabbit antibody against human cytochrome P450 2A6 were from QED Bioscience Inc. (San Diego, California). Horseradish peroxidase linked donkey anti-rabbit IgG and an enhanced chemiluminescence detection system (ECL) were from Amersham (Buckinghamshire, UK). A nitrocellulose membrane for western blotting, pore size 0.45 μm, was from BioRad, Herts, UK. All other chemicals and reagents were purchased from Sigma (Dorset, UK). ImageQuant programme was from Molecular Dynamics, GE Healthcare (Buchinghamshire, UK).

Animals. Entire male pigs of a commercial Large White crossbreed (40% Large White x 40% Landrace x 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 EU-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 used immediately for hepatocyte isolation. The effects of indole, androstenone and estrone sulphate on CYP2A6 expression were studied on four animals. The effects of skatole and testosterone were studied on five and six animals respectively. All the measurements were done in duplicate.

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Hepatocyte isolation and culture. Hepatocytes were isolated as described in Doran et al. (2002b). Cell viability was determined by trypan blue exclusion and was approximately 90%. The isolated hepatocytes were plated on collagen-coated plates (approximately 6 x 10^6 cells per plate) with 10 ml of Medium 199. The cells were pre-incubated for 24 h at 37 °C in a humidified atmosphere or air (95%) and CO_2 (5%) to ensure attachment of the hepatocytes to the plates. After 24 h the media was replaced with a fresh media with or without treatment (experimental and control plates respectively) and the cells were further incubated for 24 h under the same conditions. After 24 h following the treatment, the hepatocytes were washed twice with phosphate buffered saline (PBS), scraped into 0.3 ml of PBS with added inhibitors of proteolytic enzymes antipain + pepstatin + leupeptine (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 sulphate, 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 in order to ensure that the maximum final concentration of methanol or ethanol added to the cells was not higher than 0.5%. Adding of methanol or ethanol alone at the final concentrations up to 0.5% did not have any effect on CYP2A6 protein expression.

Analyses of CYP2A6 protein expression. CYP2A6 protein expression in the primary cultured hepatocytes was analysed by Western blotting following the procedure described previously (Nicoulau-Solano et al., 2006) with minor modification. The cell proteins (6 µg) were separated by SDS-PAGE, electroblotted onto a nitrocellulose membrane and probed with primary antibody (rabbit anti-human polyclonal CYP2A6), which were used at a dilution of 1:2000. It has been previously reported by immunoblotting that the antibodies against human CYP2A6 cross-react with the pig hepatic CYP2A6 protein (Soucek et al., 2001). After probing with primary antibody, the blot was washed with PBST and re-probed

with commercial secondary antibody, which were used in the dilution 1:10,000. The blots were developed with ECL reagent. A clear band of an approximately 50 kDa which corresponds to the molecular weight of pig hepatic CYP2A6 (Lin et al., 2004), was detected. The intensity of the signals was quantified using ImageQuanto program (Molecular Dynamics, GE HealthCare UK Ltd, Buckinghamshire, UK). Differences between CYP2A6 protein expression in control hepatocytes and hepatocytes incubated in the presence of steroids, indole or skatole was analysed using Student's *t*-test. $P < 0.05$ was considered statically significant.

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 when compared to the control values. There were no statically significant differences between the CYP2A6 protein levels in the presence of 1, 10 and 100 nM of skatole. Further increases in skatole concentration to 500 nM and 1000 nM were accompanied by decrease in CYP2A6 protein expression, which returned to the control values in the presence of 1000 nM of 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 when compared to similar concentrations of skatole. Thus, expression of CYP2A6 protein in the presence of 1, 10 and 100 nM of indole was 105%, 131% and 145% higher when compared to 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 sulphate on CYP2A6 protein expression in primary cultured hepatocytes is presented in Fig. 3-5.

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, when compared to 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 increase to 500 and 1000 nM.

In contrast to androstenone, incubation with testosterone and estrone sulphate did not have inhibitory effects on CYP2A6 protein expression at any of the concentrations studied (Fig. 4 and 5). Moreover, there was tendency for an increase in CYP2A6 protein level in the presence of 1 to 100 nM estrone sulphate (Fig. 5) and 500 and 1000 mM of 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 cytochrome P450 system (CYP450), in particular the isoforms CYP2E1 and CYP2A6 (Babol et al., 1998; Diaz and Squires, 2000). Low activity and expression of these CYP450s leads to a reduced rate of hepatic skatole metabolism followed by skatole accumulation in pig adipose tissue (Diaz and Squires, 2000; Doran et al., 2002a).

In other species skatole metabolism might take place in tissues other than the liver. For example, in ruminants skatole is intensively metabolised 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 catalysed by the cytochromes of 2F and 2B sub-families (Ramakanth et al., 1994; Carr et al., 2003).

There is no information on whether skatole can be metabolised in extra-hepatic 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, whist CYP2A6 mRNA was only present in liver and kidney. The expression of both, CYP2E1 and CYP2A6 mRNA was much higher in liver when compared to 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., 2002b). 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., 2002b). 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 CYP450s expression in isolated cultured rat hepatocytes (Woodcroft and Novak, 1997; Wu et al, 1997).

In the present study we have observed a bifisal 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 bifisal response of CYP450 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 porphyrinogenic 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 an 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 those previously reported for the induction of CYP2E1 protein expression by skatole (by 45%) (Doran et al., 2002b). The mechanisms of CYP2A6 and CYP2E1 protein induction by skatole are unknown. According to the literature, the classical CYP2A6 inducers (i.e. phenobarbitale, rifampicin, pyrazol) act at the mRNA level (Donato et al. 2000). Further 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 CYP450s. It needs to be further investigated whether the induction of the porcine CYP2A6 and CYP2E1 protein expression by skatole and indole are related to an increase in their mRNA levels.

In respect to the testicular steroids, the present study has established that only androstenone (not testosterone or estrone sulphate) 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 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., 2002b). Whether this androstenone-mediated decrease in CYP2A6 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 increase to 500 nM and 1000 nM. The mechanism of this bifasal response of CYP2A6 protein expression to androstenone treatment is unknown.

In contrast to androstenone, testosterone did not have any 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 might be 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 possible some other sex steroids) on the expression of the skatole-metabolising 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 sulphate in plasma and fat (Babol et al., 1999, Zamaratskaia et al., 2005). Although, the present study did not find any significant effect of estrone sulphate on the expression of CYP2A6 protein in primary cell hepatocytes, it does

not exclude that estrone sulphate directly effects CYP2A6 activity without influencing CYP2A6 expression.

This study is the first communication which has investigated the role of testicular steroids and non-steroid boar taint compounds on the expression of pig hepatic CYP2A6. The main finding of this research is that CYP2A6 protein expression can be induced by physiological concentrations of skatole and indole and inhibited by androstenone in primary cultured hepatocytes. The results of this study contribute to understanding the mechanisms regulating the expression of pig hepatic CYP2A6.

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Footnotes

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Legends for figures

Figure 1. Effect of skatole on CYP2A6 protein expression in primary cultured pig hepatocytes. Hepatocytes were pre-incubated for 24 hours prior to the addition of skatole at one of the following final concentrations: 1, 10, 100, 500 or 1000 nM. After the addition of skatole the hepatocytes were incubated for a further 24 h. Control cells (skatole concentration 0) were treated similarly to the experimental cells except no skatole was added. CYP2A6 protein levels in control cells were taken as 100 arbitrary units. Each bar represents the average values for five cell preparations from five pigs. Analysis of CYP2A6 expression in each cell preparation was preformed in duplicate. The error bars represent the S.E.M.

$^{*}p < 0.05$; $^{***}p < 0.001$.

Figure 2. Effect of indole on CYP2A6 protein expression in primary cultured pig hepatocytes. Hepatocytes were pre-incubated for 24 hours prior to the addition of indole at one of the following final concentrations: 1, 10, 100, 500 or 1000 nM. After the addition of indole the hepatocytes were incubated for a further 24 h. Control cells (indole concentration 0) were treated similarly to the experimental cells except no indole was added. CYP2A6 protein levels in control cells were taken as 100 arbitrary units. Each bar represents the average values for four cell preparations from four pigs. Analysis of CYP2A6 expression in each cell preparation was preformed in duplicate. The error bars represent the S.E.M.

$^{*}p < 0.05$; $^{**}p < 0.01$.

Figure 3. Effect of androstenone on CYP2A6 protein expression in primary cultured pig hepatocytes. Hepatocytes were pre-incubated for 24 hours prior to the addition of androstenone at one of the following final concentrations: 1, 10, 100, 500 or 1000 nM. After the addition of androstenone the hepatocytes were incubated for a further 24 h. Control cells

(androstenone concentration 0) were treated similarly to the experimental cells except no androstenone was added. CYP2A6 protein levels in control cells were taken as 100 arbitrary units. Each bar represents the average values for four cell preparations from four pigs. Analysis of CYP2A6 expression in each cell preparation was preformed in duplicate. The error bars represent the S.E.M. $^{*}p < 0.5$; $^{**}p < 0.01$; $^{***}p < 0.001$.

Figure 4. Effect of testosterone on CYP2A6 protein expression in primary cultured pig hepatocytes. Hepatocytes were pre-incubated for 24 hours prior to the addition of testosterone at one of the following final concentrations: 1, 10, 100, 500 or 1000 nM. After the addition of testosterone the hepatocytes were incubated for a further 24 h. Control cells (testosterone concentration 0) were treated similarly to the experimental cells except no androstenone was added. CYP2A6 protein levels in control cells were taken as 100 arbitrary units. Each bar represents the average values for cell six preparations from six pigs. Analysis of CYP2A6 expression in each cell preparation was preformed in duplicate. The error bars represent the S.E.M.

Figure 5. Effect of estrone sulphate on CYP2A6 protein expression in primary cultured pig hepatocytes. Hepatocytes were pre-incubated for 24 hours prior to the addition of estrone sulphate at one of the following final concentrations: 1, 10, 100, 500 or 1000 nM. After the addition of estrone sulphate the hepatocytes were incubated for a further 24 h. Control cells (estrone sulphate concentration 0) were treated similarly to the experimental cells except no estrone sulphate was added. CYP2A6 protein levels in control cells were taken as 100 arbitrary units. Each bar represents the average values for four cell preparations from four pigs. Analysis of CYP2A6 expression in each cell preparation was preformed in duplicate. The error bars represent the S.E.M.

Figure 1

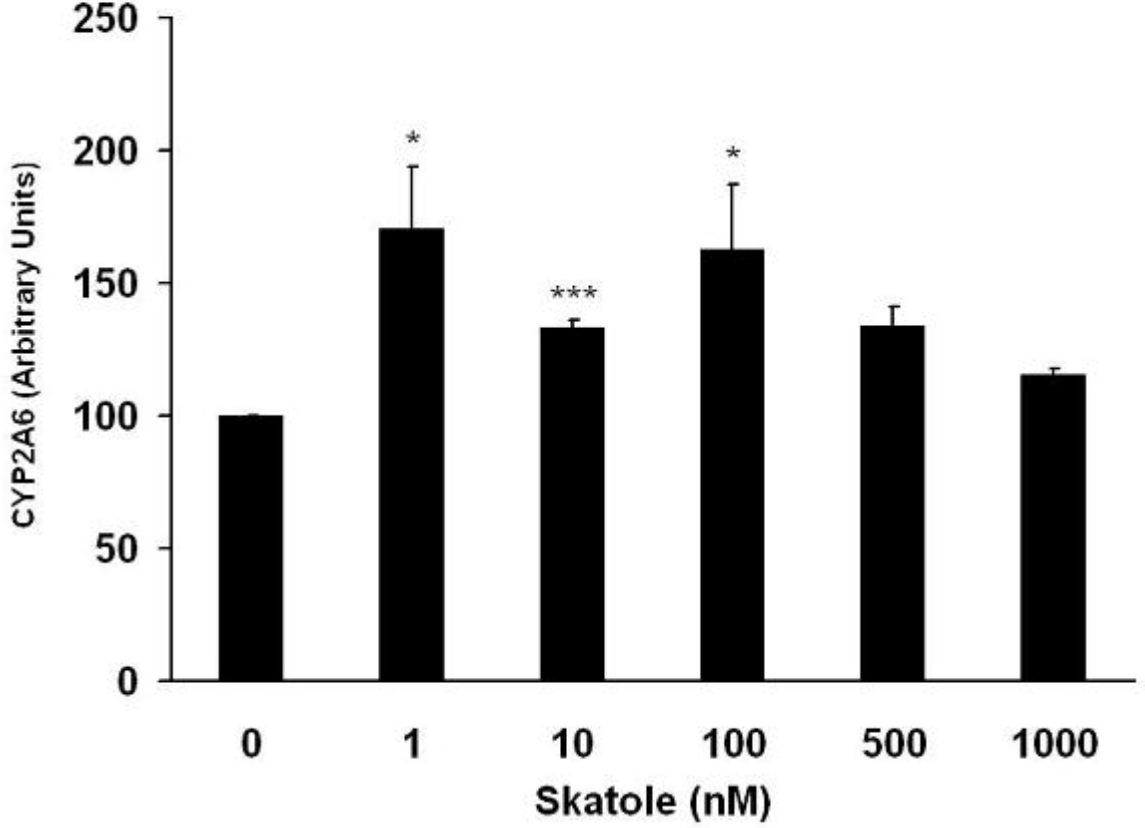

250
200
150
100
50
0
CYP2A6 (Arbitrary Units)
*
***
*
0
1
10
100
500
1000
Skatole (nM)

Figure 2

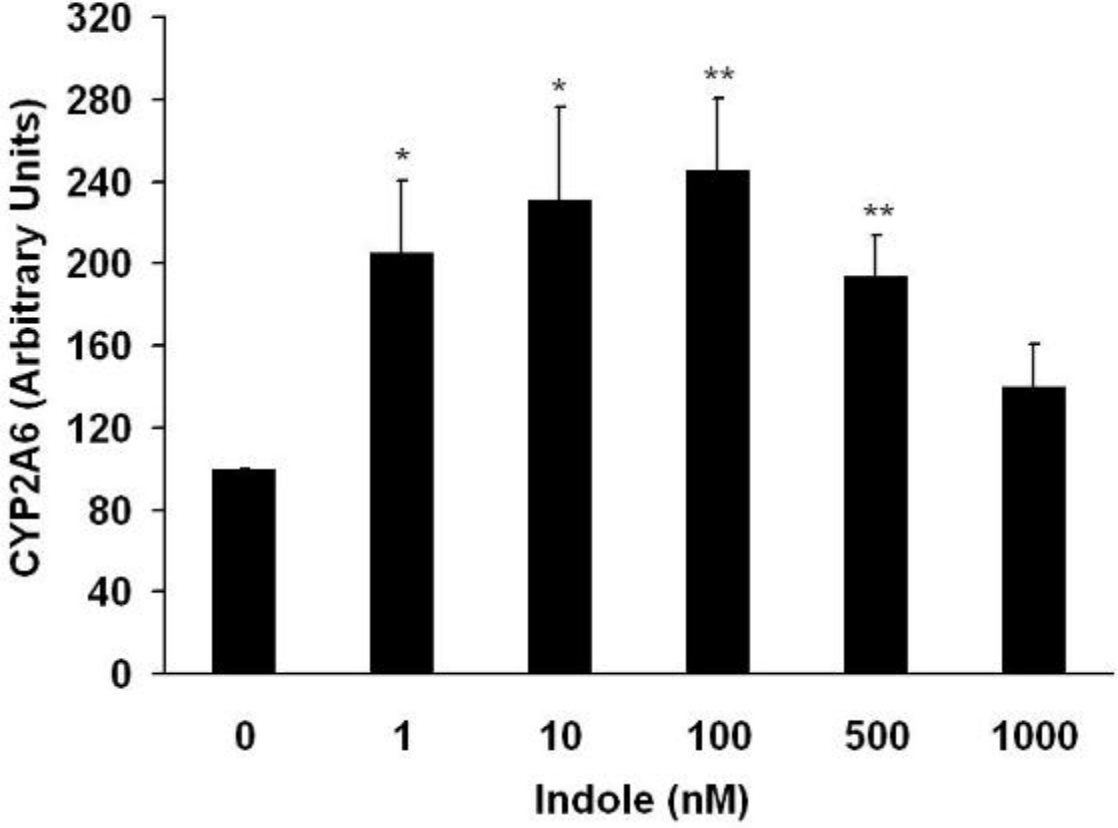

320
280
240
200
160
120
80
40
0
CYP2A6 (Arbitrary Units)
*
*
**
**
0
1
10
100
500
1000
Indole (nM)

Figure 3

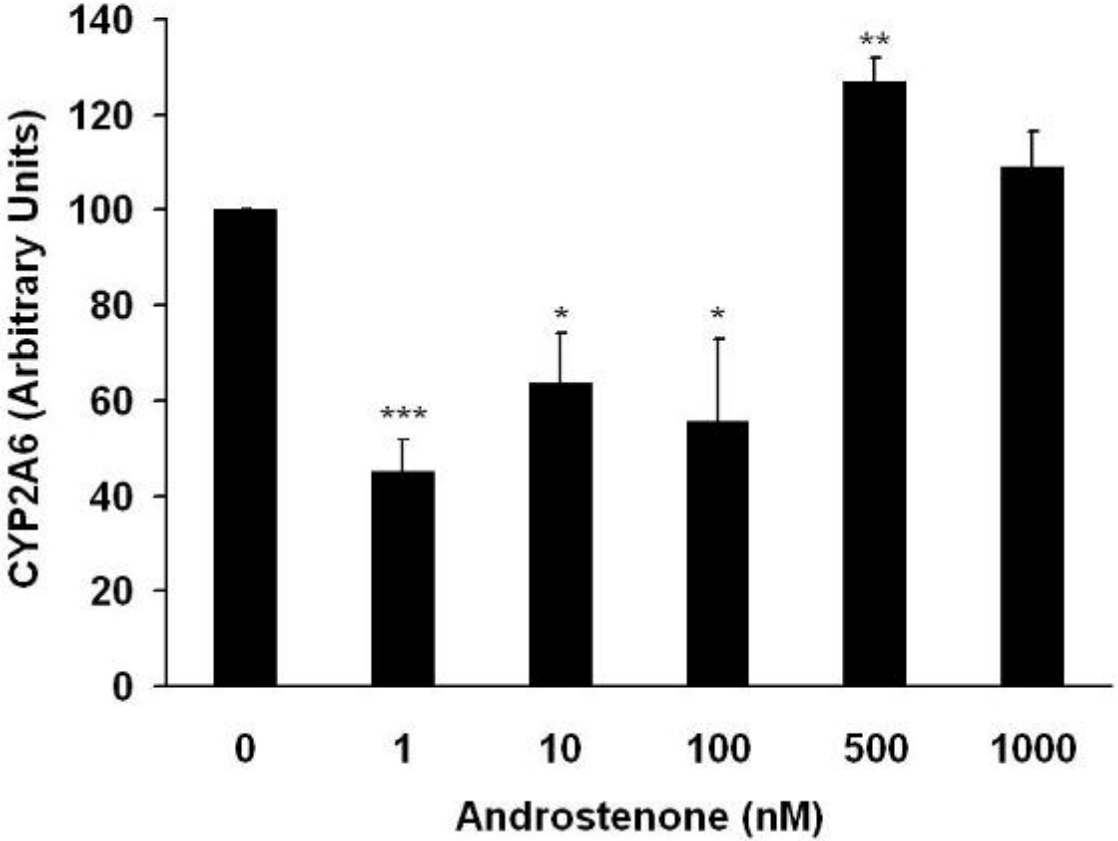

140
120
100
80
60
40
20
0
CYP2A6 (Arbitrary Units)
***
*
*
**
0
1
10
100
500
1000
Androstenone (nM)

Figure 4

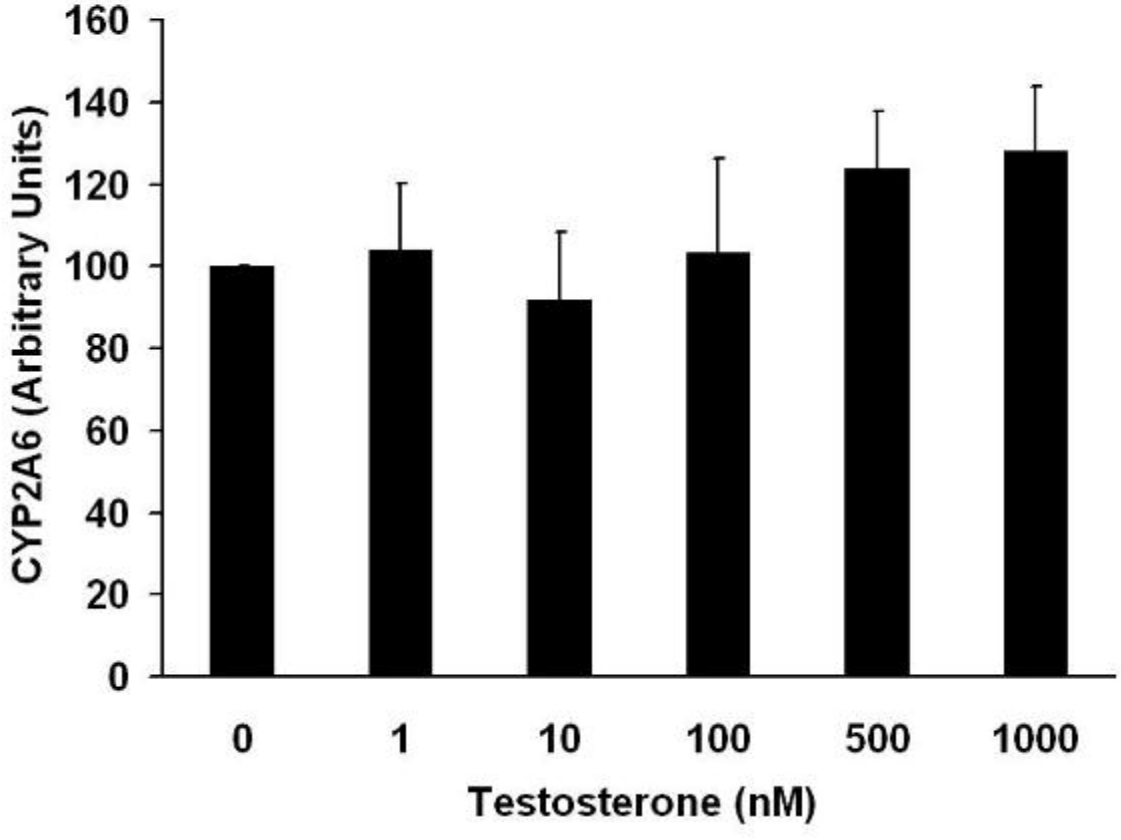
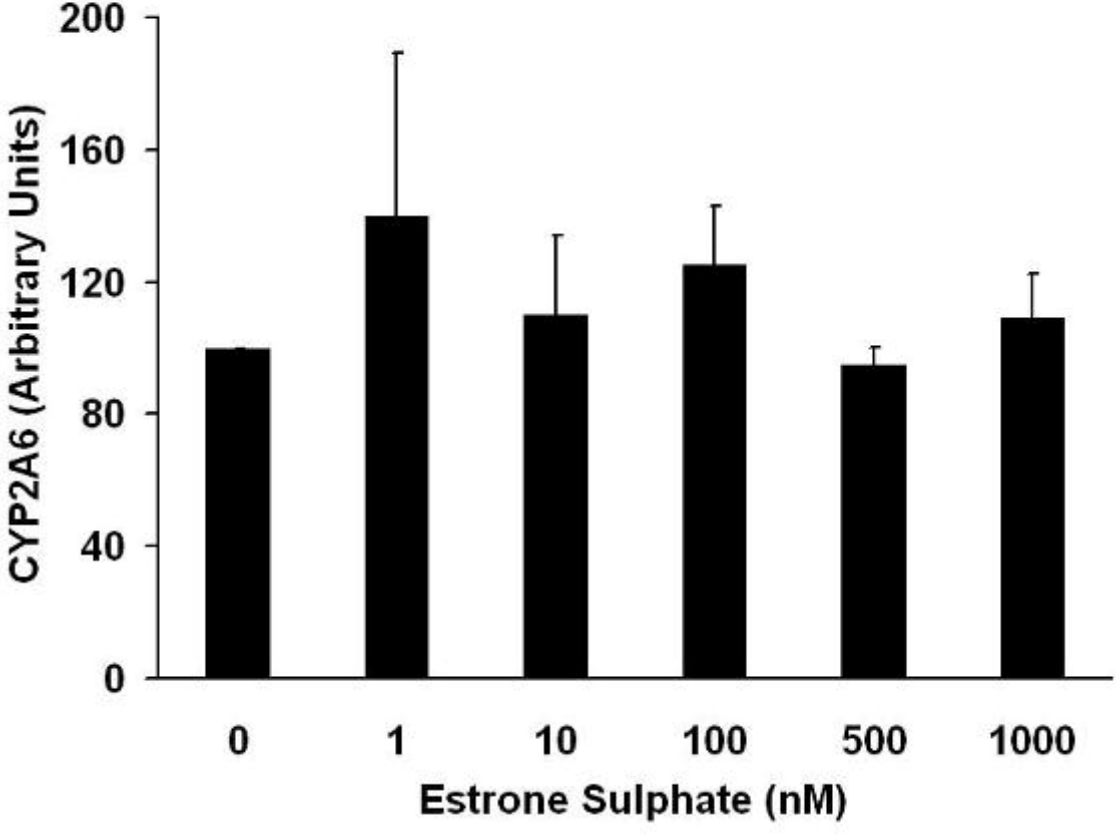

160
140
120
100
80
60
40
20
0
CYP2A6 (Arbitrary Units)
0
1
10
100
500
1000
Testosterone (nM)

Figure 5


200
160
120
80
40
0
CYP2A6 (Arbitrary Units)
0
1
10
100
500
1000
Estrone Sulphate (nM)