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Title Page

THE ROLE OF CYP2A AND CYP2E1 IN THE METABOLISM OF 3-METHYLINDOLE IN PRIMARY CULTURED PORCINE HEPATOCYTES

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Running Title Page

METABOLISM OF 3-METHYLINDOLE BY P450s IN PORCINE HEPATOCYTES

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ABBREVIATIONS: 3MI, 3-methylindole; 3MOI, 3-methyloxindole; 4MP, 4-methylpyrazole; 8MOP, 8-methyoxypsoralen; ABT, 1-aminobenzotriazole; COH, coumarin 7-hydroxylase; DDTC, diethyldithiocarbamate; HMOI, 3-hydroxy-3-methyloxindole; KCZ, ketoconazole; PNC, *p* – nitrocatechol; PNP, *p* – nitrophenol

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Abstract

The accumulation of 3-methylindole (3MI) in uncastrated male pigs (boars) is a major cause of boar taint, which negatively affects the quality of meat from the animal. Previously, CYP2E1 and CYP2A have been identified as cytochrome P450 isoforms involved in the metabolism of 3MI using porcine liver microsomes. This study further examines the role of these isoforms in the metabolism of 3MI using a primary porcine hepatocyte model, by examining metabolic profiles of 3MI following incubation with P450 inhibitors. Incubation of hepatocytes with 4-methylpyrazole resulted in a selective inhibition of CYP2E1 activity as determined by *p*-nitrophenol hydroxylase activity, and an associated significant decrease in the production of the 3MI metabolites; 3-hydroxy-3-methyloxindole and 3-methyloxindole. Furthermore, inhibition of CYP2A, as assayed by coumarin 7-hydroxylase activity, using 8-methoxypsoralen and diethyldithiocarbamate was not associated with any further significant inhibition of the production of 3MI metabolites. Treatment with general P450 inhibitors resulted in further decreases in CYP2E1 activity and a more dramatic decrease in the production of 3MI metabolites, suggesting that additional P450s may be involved in the phase 1 metabolism of 3-methylindole. In conclusion, CYP2E1 activity levels are more important than CYP2A activity levels for the metabolism of 3-methylindole in isolated pig hepatocytes.

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Introduction

3-methylindole (3MI) is a pneumotoxin in several mammalian species including goats, cattle and humans (Yost, 1989). In mature uncastrated male pigs (boars), 3MI causes a problem of boar taint, where high levels of the compound present in the adipose tissue release an unpleasant odour when the meat of boars is heated. As a consequence, male pigs are routinely castrated for meat production. 3MI is produced from the bacterial degradation of tryptophan in the hindgut of the pig and is metabolized in the liver to more readily excretable forms (Bonneau, 1997). The two most important control points that affect the accumulation of 3MI include its production and subsequent absorption from the hind gut and the metabolism of 3MI in the liver to more readily excretable forms (Claus et al., 1994; Squires and Bonneau, 2004). The formation of particular 3MI metabolites has been shown to be an important factor in 3MI clearance (Friis, 1992). In addition, various environmental and management factors can also affect 3MI levels in the pig.

Many studies have investigated the role of cytochrome P450 in the hepatic metabolism of 3MI in various species (Thornton-Manning et al., 1996; Squires and Lundström, 1997; Lanza and Yost, 2001). Huijzer et al. (1989) demonstrated, using suicide substrates for P450s in goat microsomes, that bioactivation of 3MI and covalent binding resulting in toxicity is mediated by P450 enzymes. Pigs are not susceptible to 3MI toxicity, suggesting that pigs metabolize 3MI by different P450 enzymes than other susceptible species. In a study conducted by Diaz and Squires (2000a), the two isoforms of P450 suggested to play a key role in the biotransformation of 3MI in pig liver microsomes were CYP2A and CYP2E1. Prior to this study, CYP2E1 was the only P450 isoform that was shown to metabolize 3MI in pigs (Friis, 1995; Babol et al., 1998; Squires and Lundström, 1997). Doran et al. (2002) has used primary porcine hepatocytes to investigate the effect of 3MI and androstenone (the other major contributor to boar taint in pigs) on CYP2E1 activity. In this present study, we used the isolated porcine hepatocyte model to

elucidate the relative importance of CYP2A and CYP2E1 and potentially additional P450 involvement in the metabolism of 3MI.

Three-week-old male pigs were used as a source for hepatocytes since they have similar hormonal profiles compared to sexually mature adult males (Schwarzenberger et al., 1993). Given that castration decreases 3MI levels and essentially eliminates the problem of boar taint (Babol et al., 1996), it can be inferred that a testicular factor is responsible for either the production or metabolism of 3MI, and thus hormone levels are very relevant in the animal model chosen for these experiments. Hepatocytes have the advantage over microsomes of being an inducible *in vitro* system where enzyme protein and activity levels can be manipulated by genetic regulation.

Enzyme activities for CYP2A and CYP2E1 were measured in this study by the hydroxylation of specific substrates, coumarin and *p*-nitrophenol, respectively (Skaanild and Friis, 2005; Baranova et al., 2005), and inhibitors were used to determine the importance of these CYP isoforms in 3MI metabolism. 8MOP is used as both an *in vitro* and *in vivo* inhibitor of CYP2A6 activity in humans (Sellers et al., 2000; Zhang et al., 2001), whereas 4MP has been used as a CYP2E1-specific inhibitor in several *in vitro* studies, including in rat liver microsomes (Turini et al., 1998), in primary cultured rat hepatocytes (Ferrara et al., 2001), human liver microsomes (Spracklin et al., 1997), monkey liver microsomes (Amato et al., 1998) and pig liver microsomes (Diaz et al, 2000a). In addition DDTC, a metabolite of disulfiram, has been used to inhibit CYP2E1 activity by irreversible, mechanism-based inhibition in microsomes of multiple species including pigs (Court et al., 1997), but may also affect CYP2A levels since many CYP2E1 substrates can be oxidized by CYP2A6 (Pass and Mclean, 2002). Disulfiram has been reported to not inhibit CYP2A6 activity in humans after a single dose (Kharash et al., 1998); however, the specificity of these P450 inhibitors has not been previously demonstrated in the pig. The involvement of additional P450 isoforms was also investigated by using general inhibitors that target total P450 activity. General inhibitors have previously been used to

determine the effect of monooxygenation on the metabolism of 3MI in other species (Huijzer et al., 1989; Ruangyuttikarn et al., 1991). Our objective was to evaluate the effect of these inhibitors on the activity of CYP2E1 and CYP2A and the metabolism of 3MI in isolated porcine hepatocytes.

Methods

Chemicals

4-Methylpyrazole (4MP), 8-methoxysporalen (8MOP), diethyldithiocarbamate (DDTC), ketoconazole (KCZ), 1-aminobenzotriazole (ABT), 3-methylindole, indole-3-carbinole, 2-aminoacetophenone (2AAP) and β-NADPH were obtained from Sigma (Oakville, ON). The 3MI metabolite standards 3-methyloxindole (3MOI) and 3-hydroxy-3-methyloxindole (HMOI) were a gift provided by Dr. G. S. Yost, Department of Pharmacology and Toxicology, University of Utah (Salt Lake City, UT) and 5-hydroxy-3-methylindole was a gift provided by Jens Hansen Møller of the Danish Meat Research Institute (Roskilde, Denmark). Type I collagenase (activity 274 U/mg) was purchased from Worthington Biochemical Corporation (Lakewood, NJ). Iletin® regular insulin (beef and pork) was purchased from Eli Lilly Company (Indianapolis, IN). All remaining reagents were acquired from Sigma (Oakville, ON).

Research Animals

All animals used for these experiments were obtained from Arkell Swine Research facility of the University of Guelph and used in accordance with the guidelines of the Canadian Council on Animal Care. Pigs were uncastrated Yorkshire males at 21 ± 1 days of age at time of slaughter. Immediately following slaughter, livers were surgically excised and used for liver perfusions or frozen in liquid nitrogen and stored at –70 ºC for future microsomal analysis. For microsomal enzymatic assay analysis, livers were obtained from sexually mature uncastrated Yorkshire males (boars) and from age-matched adult castrated males (barrows).

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Microsomal Preparation

For microsomal analysis, frozen liver samples were homogenized in a Polytron homogenizer in 2 x (w/v) homogenizing buffer containing 50 mM Tris-HCl, 150 mM KCl, 250 mM sucrose and 1 mM EDTA (pH7.4) for 1 minute on ice. Samples were centrifuged at 9000 x g for 30 minutes at 4 ºC. The supernatant was re- centrifuged at 105 000 x g for 60 minutes at 4 ºC. Following ultracentrifugation, the microsomal pellet was suspended in 1x (w/v) of storage buffer containing 50 mM Tris-HCl, 20% glycerol and 1 mM EDTA (pH 7.4). The microsomal protein content was then determined by the Bradford method using the Bio-Rad Protein Assay Reagent (Bio-Rad Laboratories Inc., Mississauga, ON) and aliquots were frozen at –70 ºC until needed.

Liver Perfusion and Hepatocyte Culture

Hepatocytes were prepared by a modification of a collagenase perfusion method described previously (Sinclair et al., 2005). Briefly, one hepatic lobe was immediately catherterized by selecting the largest vessel at the cut end. Blood was then flushed from the liver by perfusion with 25 mL/min of medium containing 10 mM hydroxyethyl piperazine ethane (HEPES), 1 mM EGTA and Hanks' Balanced Salt Solution (pH 7.4) delivered at 37 ºC. The lobe was then perfused for 5 minutes with the same medium without EGTA and then with William's E Media (pH 7.4) containing 10 mM HEPES and 0.7 mg/mL collagenase type 1 for approximately 15 minutes until the hepatocytes appeared to be fully dissociated.

All remaining experimental procedures were carried out under sterile conditions. The lobe was gently minced with a scalpel to disassociate cells in attachment medium containing 10 mM HEPES, 10% (v/v) fetal bovine serum, 1% (v/v) penicillin-streptomycin and 50 Units/L insulin in William's E media (pH 7.4), at 4 ºC. Cells were filtered through gauze and centrifuged at 15 x g for 3 minutes. The cell pellet was washed in attachment media and cell viability was determined by trypan blue exclusion. Hepatocytes were only used if viability was greater than

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80%. Hepatocytes were then plated on Primaria™ 60 mm tissue culture dishes (VWR International Ltd., Mississauga, ON) at a density of 2.25 x 10^6 cells in 3.0 mL attachment media, and kept in a incubator at 37ºC supplied with 95% air and 5% CO_2. After 4 hours of attachment, the medium was replaced with serum-free medium that contained 10 mM HEPES, 10 mM pyruvate, 0.35 mM proline, 1% (v/v) penicillin-streptomycin and 50 Units/L insulin in William's E media (pH 7.4). Inhibitors used to treat the hepatocytes were dissolved in methanol and used at a final methanol concentration of less than 0.05 %. Following 1 hour of pre-incubation with inhibitors, the P450 activity and 3MI metabolism studies were carried out. Cell mortality was assessed by cell detachment from tissue culture dishes (Smets et al., 2002).

p-Nitrophenol Hydroxylase Activity Assay

To determine the rate of p-nitrophenol (PNP) hydroxylase activity, primary hepatocytes were incubated with 500 µM PNP for 6 hrs. PNP hydroxylase activity was proportional to number of cells, and linear with time over eight hours (R^2 = 0.99). After incubation, media was added to an equal volume of acetonitrile and frozen until analysis. After thawing, the samples were vortexed briefly and centrifuged at 15 000 x g for 20 minutes to remove protein before HPLC analysis. A 100 µL aliquot of the supernatant was injected using a Spectra-Physics model SP8880 (Spectra-Physics, San Jose, CA) auto sampler into a Spectra Physics HPLC consisting of a model SP8800 Ternary HPLC Pump equipped with a Spectra-Physics 100 variable wavelength detector set at 345 nM, and a Spectra-Physics model 4290 integrator. Compounds were separated on a Luna 5µ 250 x 4.60 mm reverse-phase C-18 column (Phenomenex, Torrance, CA). Elution of the compounds was accomplished using the following step-wise gradient of 100% acetonitrile (buffer A) and 25% acetonitrile, 75% double distilled water, 0.1% trifluoroacetic acid (buffer B) at a flow rate of 1.0 mL/min; 0-10 min 100% buffer B, 11-16 min 100% buffer A and 16.1-20 min 100% buffer B. Typical retention times for p-nitrocatechol (PNC) and PNP were 12 and 17 minutes respectively.

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The PNP hydroxylase assay in microsomes was adapted from Jiang et al. (1998). In a total volume of 250 uL, 1.0 mg of microsomal protein was incubated with 200 µM PNP in 0.1 M potassium phosphate buffer (pH 6.8). The mixture was allowed to equilibrate for 5 minutes at 37 ºC and reactions were carried out with 1 mM NADPH for 30 minutes at 37 ºC. The reaction was terminated by the addition of 20 µL of 40% trichloroacetic acid. Tubes were centrifuged at 15 000 x g for 15 minutes to precipitate the protein and 100 µL of the supernatant used for HPLC analysis of PNC. PNC concentration was determined with a standard curve of PNC of 0, 1, 5, 10, 20 and 40 nmol in assay buffer and activity was reported as pmol PNC per minute per mg microsomal protein.

Coumarin 7-Hydroxylase (COH) Activity

The 7-hydroxylation of coumarin was used to measure CYP2A activity, by a procedure described for cell culture by Donato et al. (1998a). Primary hepatocytes were treated with 100 µM of coumarin for 1 hour. To determine the amount of 7-hydroxycoumarin produced, 70 µL of medium was collected from each culture dish into a 96 well plate. To each well, 20 µL of 0.1 M of sodium acetate buffer (pH 4.5) containing 15 Fishman Units of β-glucuronidase and 120 Roy Units of arylsulphatase (Roche Applied Science, Laval, QC) was added, and incubated for 1 hour at 37 ºC. Then 170 µL of 0.1 M Tris-HCl (pH 9.0) was added to each well and fluorescence was measured at an excitation of 360 nm and emission at 460 nm using a Victor^{3TM} plate reader (PerkinElmer Life Sciences, Woodbridge ON) equipped with the Wallac 1430 (version 3.0) software. The COH assays were linear with number of cells, microsomal protein content and with time.

For the microsomal analysis, a total of 0.8 mg of microsomal protein was combined with 0.2 mM coumarin, 5 mM magnesium chloride in 50 mM Tris buffer (pH 7.4) in a volume of 200 µL. After 5 minutes of preincubation, the reaction was started by the addition of 25 µL of 25 mM NADPH and incubated for 15 minutes at 37 ºC. The reaction was stopped by the addition of

100 µL of 20% trichloroacetic acid, followed by a 5 minute centrifugation at 10 000 x g to pellet protein. A total of 200 µL of the supernatant was added to 1.5 mL of 0.1 M Tris buffer (pH 9.0) and the fluorescence was determined using a Hitachi F2000 Fluorescence Spectrophotometer (Hitachi, Japan) with an excitation of 360 nm and emission at 460 nm. The concentration of 7-hydroxycoumarin formed was determined by interpretation of a standard curve and reported as pmol 7-hydroxycoumarin per minute per mg microsomal protein.

3MI Metabolism Assay

To measure 3MI metabolism in primary cultured hepatocytes, cells were incubated with 500 µM 3MI for 6 hours, after which 500 µL of medium was added to an equal volume of acetonitrile to stop the reaction. The extract was centrifuged at 15 000 x g for 20 minutes to pellet protein and a 100 µL aliquot was used for HPLC analysis, using the chromatograph previously described, with the UV detector set at 250 nM, and a SpectroSystem fluorescence FL3000 (Thermo Separation Products, Fremont, CA) detector with the wavelength setting at 285 nm excitation, 350 nm emission. Metabolites were separated on a Luna 5µ 250 x 4.60 mm reverse-phase C-18 column (Phenomenex, Torrance, CA). The metabolites were eluted using the following gradient of 100% acetonitrile (buffer A) and 0.01 M potassium dihydrogen phosphate pH 3.9 (buffer B) at a flow rate of 1.2 mL/min; 0.0 min 10% buffer A, 6.0 min 20% buffer A, 12.0 min 30% buffer A, 25.0 min 70% buffer A, 25.1 min 100% buffer A, 30.0 min 100% buffer A, 30.1 - 35 min 10% buffer A. A mixture of standards was used after every 9th sample on the HPLC for identification of metabolite peaks. Concentration curves for metabolites up to 300 ng/ml were used to calculate pmol of metabolites produced. Standards, including its presence in cells, concentration of standard used and typical retention times are presented in Table I.

Inhibition Experiments

Inhibitors were used to elucidate the role of the isoforms, CYP2A and CYP2E1 in the metabolism of 3MI. To achieve inhibition of CYP2E1 and CYP2A, the inhibitors 4MP, 8MOP and DDTC were used as treatment groups. Following attachment of hepatocytes, 50 μM of 4MP, 200 μM of 8MOP or 250 μM of DDTC was added in 3 mL of serum-free medium. For general inhibition of P450s, the inhibitors KCZ and ABT at a concentration of 250 μM were used. After a 1-hour incubation period with the inhibitors, PNP and COH activity assays, and the 3MI metabolism assay were performed.

Statistical Analysis

The model used for the treatment effect of inhibitor on enzyme activity and 3MI metabolite production was as follows:

$$Y_{ijkl} = \tau_i \times \pi_{j(i\,k)} \times \beta_k \times \beta x \tau_l \times \varepsilon_{ijkl}$$

Where Y_{ijkl} = chromatogram peak, τ_i = inhibitor treatment, $\pi_{j(i\,k)}$ = plate nested within inhibitor and within liver, β_k = liver, $\beta x \tau_l$ = liver x inhibitor interaction and ε_{ijkl} = the residual error of the experiments. Significant differences in PNP conversion, COH activity and 3MI metabolism were determined using the Tukey's test for pairwise comparison in the Proc mixed program of the Statistical Analysis Software v8.0 (SAS institute, Carry, NC). Treatment related differences were determined with inhibitor as fixed effects and liver (treated as a block), liver x inhibitor interaction and plate within inhibitor within liver as random effects. The Satherthwaite approximation was used to calculate the denominator for the degrees of freedom given that variances were pooled for analysis. Activity and concentration of 3MI metabolite data was either $\log_e$ or square root transformed to meet ANOVA assumptions of residual normality and homogeneous variance. The resulting analysis was examined using the Shapiro-Wilkes' test (Proc univariate normal) and by graphical interpretation of the residual plots. For all comparisons a type I error rate of $\alpha = 0.05$ was used unless otherwise stated. All results are

presented as means ± standard errors, while all graphs are presented using the normalized raw data for easier illustration, with statistical results and interpretations performed using the transformed data.

For microsomal enzyme activity analysis significant differences were examined with a Tukey's test using the Proc GLM program of the SAS program and a type I error rate of $\alpha = 0.05$. All linear regressions were calculated using Microsoft Excel.

Results

CYP2E1 and CYP2A Activity in Microsomes

In order to determine the suitability of using hepatocytes from 3-week old male pigs as a model of the adult boar liver, we measured microsomal P450 activity levels. Figure I illustrates the activity level comparison in microsomes for CYP2A and CYP2E1 between sexually mature boars, adult castrated males and uncastrated 3-week old males. CYP2E1 and CYP2A activities (as determined by PNP hydroxylase and COH activities respectively) in 3-week old males were higher than the levels observed in boars and lower than barrows. All enzyme levels were significantly different from one another ($P<0.0001$). Thus, the 3-week old male pig represents a better model for investigating 3MI metabolism in the adult boar compared to the adult castrated male pig.

3MI Metabolite Profile in Primary Hepatocytes

Three metabolites were identified following incubation of hepatocytes with 3MI (Table I). The time course for the production of metabolites was investigated following attachment of the cells by incubating 15 culture dishes with 500 µM 3MI in serum-free medium and sampling from 3 plates at 0, 4, 8, 16 and 24 hours incubation time. The production of HMOI and 3MOI were linear over 24 hours of incubation of cells with 3MI with a rate of 2.7 pmol/million cells/hr (r^2 = 0.96) and 1.1 pmol/million cells/hr (r^2 = 0.97) respectively. Production of the minor metabolite, 2-aminoacetophenone (2-AAP) was linear within 4 hours of treatment with 3MI and then plateaued.

P450 inhibition experiments

While chemical inhibitors have previously been shown to have an affect on 3MI metabolism in microsomal assays, we wanted to determine whether these inhibitors would

result in changes in the 3MI metabolism profile in primary cultured hepatocytes. The inhibition curves of CYP2E1 and CYP2A activity with the inhibitors 4MP, 8MOP and DDTC are presented in Figure II. The inhibitor 4MP decreased CYP2E1 activity to a maximum inhibition of 94% at 100 μM concentration, while there was no significant inhibition of CYP2A activity. Therefore, 4MP was considered a specific inhibitor of CYP2E1 activity with no effect on CYP2A. 8-MOP inhibited both CYP2E1 and CYP2A activity with a maximum inhibition of 93 and 91% respectively at a concentration of 500 μM 8MOP. DDTC also decreased both CYP2E1 and CYP2A activity with a maximum inhibition of 72 and 77% respectively at a concentration of 1 mM. DDTC initially increased both activities at the lower concentrations examined (1 and 10 μM DDTC), with a sharp decrease observed at higher concentrations of the inhibitor. There was no visible cell mortality after 24 hours exposure of cultured hepatocytes with these three inhibitors.

The activity of CYP2E1 in hepatocytes from 3 pigs averaged 1.09 ± 0.23 nmol/million cells/hr. Treatments with 50 μM 4MP, 200 μM 8MOP and 250 μM DDTC resulted in a significant similar decrease in CYP2E1 activity compared to controls of 74% (P=0.0005), 72% (P=0.0006) and 64% (P=0.0017) respectively (Figure III). CYP2A activity averaged 14.72 ± 4.52 nmol/million cells/hr in hepatocytes. CYP2A activity was significantly inhibited in the 8MOP and DDTC treatment groups by 59% (P=0.003) and 72% (P=0.0005) respectively. Treatment with 4MP resulted in no significant change compared to controls (P=1.0). The production of the two major 3MI metabolites, HMOI and 3MOI averaged 2.25 and 1.26 pmol/million cells/hr respectively. The production of the metabolites HMOI and 3MOI were significantly inhibited by 24% (P=0.044) and 21% (P=0.025) respectively when cells were treated with 50 μM 4MP. Treatment with 200 μM 8MOP resulted in a decrease trend in production of 19% (P=0.10) and a 28% decrease (P=0.006) for HMOI and 3MOI respectively. Treatment with 250 μM DDTC resulted in a significant decrease in production of 36% (P=0.007) and 36% (P=0.001) for HMOI and 3MOI respectively.

General P450 Inhibition Experiment

We next used inhibitors that are reported to decrease all P450 activity levels to investigate the potential involvement of additional P450s in the metabolism of 3MI (Figure IV). Treatment of the cells with 250 μM KCZ and 250 μM ABT significantly inhibited ($P<0.0001$) CYP2E1 activity compared to controls by 97% and 96% respectively. This inhibition from KCZ and ABT represented a further 15% significant decrease ($P=0.004$, $P=0.006$) over the 81% inhibition from treatment with 4MP (Figure IV). KCZ inhibited CYP2A activity by a significant 77% ($P=0.040$) whereas, ABT had a trend to decrease CYP2A activity by 63% ($P=0.094$). Neither KCZ nor ABT showed signs of toxicity during the 7 hr incubations; however, in a parallel experiment, KCZ did show signs of limited toxicity as ascertained by an increase in cell detachment form culture plates 24 hours after exposure.

The two major 3MI metabolites, HMOI and 3MOI were used as indicators of treatment-related changes in 3MI metabolism. There was a negative trend on HMOI production after KCZ treatment compared to both the controls and 4MP treatment group, although no significant effects were observed (Figure IV). No results for HMOI could be obtained from ABT treated cells, given that the peak from ABT could not be resolved from the HMOI peak on HPLC. For the metabolite 3MOI, KCZ significantly inhibited its production by 70% ($P=0.003$) and a significant further 41% ($P=0.015$) decrease over cells treated with 4MP. ABT significantly inhibited 3MOI production by 51% ($P=0.011$) and a further 22% ($P=0.12$) decreasing trend over cells treated with 4MP.

Discussion

Improving the metabolism and subsequent clearance of 3MI has been suggested as a means of preventing 3MI accumulation in fat of pigs (Friis, 1995; Bæk et al., 1997). Therefore, it is essential to identify the role of biotransforming enzymes in the metabolism of 3MI. A role of CYP2E1 and CYP2A in the metabolism of 3MI has been suggested through the analysis of liver microsomes of pigs (Friis, 1995; Squires and Lundström, 1997; Babol et al., 1998; Diaz and Squires, 2000a). Herein, using a primary hepatocyte model, we provide evidence that CYP2E1 is of importance in the Phase 1 metabolism of 3MI by the use of an inhibitor of CYP2E1 activity but not CYP2A activity, 4MP, and by the production of 3MI metabolites, HMOI and 3MOI. Our results show that inhibition of CYP2A and CYP2E1 activity did not reduce the production of HMOI and 3MOI metabolites any further than the inhibition of CYP2E1 alone. In addition, a further reduction in the formation of HMOI and 3MOI metabolites in hepatocytes was observed upon treatment with the general P450 inhibitors ABT and KCZ which was greater than the inhibition of CYP2E1 activity, suggesting that other P450 enzymes may be involved in the metabolism of 3MI.

Hepatocytes obtained from males at 3 weeks of age were used as a model to study 3MI metabolism, as these cells have a viability of greater than 80% upon isolation, whereas adult cells were typically less viable (unpublished data). The activities of CYP2E1 and CYP2A in microsomes from 3-week old pigs were higher than in boars and lower than in castrated males. Thus, 3-week old male pigs were determined to be a more appropriate model than castrated males to study 3MI metabolism.

Initially, Bæk et al. (1997) identified several metabolites of 3MI in the plasma and urine of pigs, including 6-hydroxy-3-methylindole (6-HO-3MI), HMOI and the mercapturate adduct of 3MI, 3-[(*N*-acetylcysteine-*S*-yl)methyl] indole. Seven metabolites were identified in incubations of 3MI with pig liver microsomes by Diaz et al. (1999) and include: HMOI, 3MOI, 6-HO-3MI, 5-HO-3MI, 3-hydroxy-3-methylindolenine, indole-3-carbinol and 2-aminoacetophenone (2AAP).

Herein three of these metabolites, HMOI, 3MOI and 2AAP were found in incubations with primary porcine hepatocytes, which further supports their use as a model for the evaluation of the role of P450's in the metabolism of 3MI. However, the use of primary cultured hepatocytes is somewhat limited by changes in the expression of drug metabolizing enzymes that can occur during the culture period.

This apparent lack of detection of some metabolites of 3MI in hepatocyte preparations can be explained by accounting for the Phase 2 biotransformation of the metabolites. It is possible that those Phase 1 products were conjugated by the time that sampling had occurred, and that they might have been end products of the metabolism pathway, ready for excretion. For example, while Friis et al. (1992) identified 5- and 6-HO-3MI as major excreteable metabolites of 3MI, we were not able to detect them in intact hepatocytes. These metabolites can be readily sulfated by cytosolic sulfotransferase (SULT) enzymes. For example, we have previously shown that high level of hepatic SULT1A1 (previously termed thermostable phenol-sulfotransferase, TS-SULT) are associated with the sulfation of 5-HO-3MI and this is related to low levels of 3MI fat accumulation (Diaz and Squires, 2003). Furthermore, we have shown that the allelic variants of the SULT1A1 gene that decrease sulfation may account for the accumulation of 3MI (Lin et al., 2004). However, due to the very close retention times, we may have been unable to resolve any sulfated 6-OH-3MI produced from the large amount of 3MOI in the incubations. The metabolite 3-hydroxy-3-methylindolenine was also identified in porcine microsomes (Diaz et al., 1999), but was not observed in cultured hepatocytes. A likely reason for this outcome is that the cytosolic enzyme aldehyde oxidase is absent in microsomes but is present in intact cells and was responsible for rapidly converting 3-hydroxy-3-methylindolenine into HMOI (Smith et al., 1996; Diaz and Squires, 2000b).

The effectiveness of P450 chemical inhibitors on CYP2E1 and CYP2A activities were determined using specific enzymatic assays. The COH activity assay is an established method for estimating CYP2A6 activity in human microsomes and in hepatocytes isolated from human,

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rabbit, dog and rat livers (Donato et al., 1998a). A porcine CYP2A which has COH activity has been described and characterized (Skaanild and Friis, 2005), and is referred to as CYP2A19, which is currently the only known porcine CYP2A. The *p*-nitrophenol (PNP) conversion to *p*-nitrocatechol (PNC) has been used for *in vitro* estimation of CYP2E1 activity in rats and humans (Donato et al., 1998b; Jiang et al., 1998), and has been utilized in microsomes prepared from minipig liver (Baranova et al., 2005)

The inhibition curves (Figure II) demonstrate the difficulty in specifically inhibiting one enzyme (CYP2A versus CYP2E1) without affecting the other. These experiments demonstrated that 8MOP and DDTC are inhibitors of both CYP2A and CYP2E1, with CYP2E1 inhibition being the more potently affected. The inhibitor 8MOP was reported to coinhibit CYP2A and CYP2E1 in monkey microsomes with an IC_{50} for PNP hydroxylase activity of 155 μM (Amato et al., 1998). DDTC has also been demonstrated to coinhibit both COH and PNP hydroxylase activity in human microsomes (Spracklin et al., 1997). Consequently there is precedence for the coinhibition observed in the current research of the CYP2A and CYP2E1 activities by inhibitors that are presumed to be specific for only one of the two enzymes.

Only the inhibitor 4MP was able to selectively inhibit CYP2E1 without affecting CYP2A, while 8MOP and DDTC were inhibitors of both enzymes. The effect of CYP2A activity on 3MI metabolism was determined by subtracting the effect of inhibiting just CYP2E1 from inhibiting both enzymes concurrently. Treatment with the inhibitors 4MP, 8MOP and DDTC resulted in a decrease of 21, 28 and 36% of control in the production of 3MOI. Thus, treatment with 8MOP and DDTC decreased levels of 3MOI by 7 and 15% respectively over the inhibition due to 4MP treatment. These differences were not significant from inhibition due to 4MP treatment (P=0.54 8MOP, P=0.08 DDTC), which suggests that CYP2A is of minor consequence in the metabolism of 3MI to 3MOI. Similarly, there was a 24, 19 and 36% decrease in HMOI production by treatment with 4MP, 8MOP and DDTC respectively. Compared to the inhibition due to 4MP treatment, DDTC and 8MOP treatment resulted in no further significant inhibition. Thus, the

patterns of HMOI and 3MOI inhibition suggest that CYP2E1 activity (PNP hydroxylase) appears to be of greater importance to the metabolism of 3MI in pig liver compared to CYP2A activity (COH).

In this study, the two general P450 inhibitors, KCZ and ABT were also utilized to inhibit total P450 activity. ABT is a suicide substrate inhibitor that has been used for prolonged P450 inhibition with both *in vitro* and *in vivo* studies in rats and humans (Ruangyuttikarn et al., 1991; Meschter et al., 1994). ABT has the advantage of being safe enough to be used *in vivo*, and therefore its use in cultured hepatocytes showed no overt confounding toxicity. KCZ is a general inhibitor of P450s both *in vitro* and *in vivo* (Loose et al., 1983; Peehl et al., 2002). The results from KCZ treatment support the results obtained by ABT treatment, but should be interpreted with caution since there was some sign of limited toxicity from KCZ after 24 hours.

The general P450 inhibitor KCZ significantly decreased CYP2E1 and CYP2A activity and ABT significantly decreased CYP2E1 activity compared to the control, and inhibited CYP2E1 to a greater amount than the CYP2E1-specific inhibitor 4MP. KCZ was also able to further decrease the production of 3MOI compared to the specific CYP2E1 inhibitor 4MP. KCZ treatment caused a 16% decrease in CYP2E1 activity compared to 4MP treatment, along with a further 41% decrease in 3MOI production. Treatment with ABT resulted in a 16% additional decrease in CYP2E1 activity compared to the 4MP treated group and a trend to a further 22% decrease in 3MOI production. Although these findings are not conclusive, the disproportionate decrease in the production of the 3MOI metabolite compared to the inhibition of CYP2E1 suggests that other P450 isoforms may be involved in 3MI metabolism in the pigs. This information is of specific relevance to the understanding of the problem of boar taint.

Genetic differences responsible for the variability in 3MI metabolism and accumulation in fat can be important molecular markers for breeding programs designed to reduce 3MI levels in fat, thereby reducing taint in boars. Here we provide evidence in isolated hepatocytes that CYP2E1 is of major importance in the Phase 1 metabolism of 3MI compared to CYP2A and we

suggest that other P450's may potentially be involved in the metabolism of 3MI. This research has also highlighted the benefits of using a cell-based system over microsomes to gain insight into the importance of considering Phase 2 metabolism in the understanding of boar taint.

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References

Amato G, Longo V, Mazzaccaro A and Gervasi PG (1998) Chlorzoxazone 6-hydroxylase and *p*-nitrophenol hydroxylase as the most suitable activities for assaying cytochrome P450 2E1 in cynomolgus monkey liver. *Drug Metab Dispos* **26:**483-489.

Babol J, Squires EJ, and Gullett EA (1996) Investigation of factors responsible for the development of boar taint. *Food Research International* **28**:573-581.

Babol J, Squires EJ and Lundstrom K (1998) Hepatic metabolism of skatole in pigs by cytochrome P4502E1. *J Anim Sci* **76:**822-828.

Baranova J, Anzenbacherova E, Anzenbacher P, and Soucek P (2005) Minipig cytochrome P450 2E1: Comparison with human enzyme. *Drug Metab. Dispos*. **33:**862-865.

Bæk C, Hansen-Møller J, Friis C, Cornett C and Hansen SH (1997) Identification of selected metabolites of skatole in plasma and urine from pigs. *J Agric Food Chem* **45:**2332-2340.

Bonneau M (1997) Boar taint in entire male pigs - a review. *Pig News and Information* **18:**15-18.

Claus R, Weiler U, and Herzog A (1994) Physiological aspects of androstenone and skatole formation in the boar - a review with experimental data. *Meat Science* **38:**289-305.

Court MH, Von Moltke LL, Shader RI and Greenblatt DJ (1997) Biotransformation of chlorzoxazone by hepatic microsomes from humans and ten other mammalian species. *Biopharm Drug Dispos* **18:**213-226.

Diaz GJ, Skordos KW, Yost GS and Squires EJ (1999) Identification of phase 1 metabolites of 3-methylindole produced by pig liver microsomes. *Drug Metab Dispos* **27:**1150-1156.

Diaz GJ and Squires EJ (2000a) Metabolism of 3-methylindole by porcine liver microsomes: Responsible cytochrome P450 enzymes. *Toxicol Sci* **55:**284-292.

Diaz GJ and Squires EJ (2000b) Role of aldehyde oxidase in the hepatic *in vitro* metabolism of 3-methylindole in pigs. *J Agric Food Chem* **48:**833-837.

Diaz GJ and Squires EJ (2003) Phase II *in vitro* metabolism of 3-methylindole metabolites in

porcine liver. *Xenobiotica* **33**:485-98.

Donato MT, Castell JV and Gómez-Lechón MJ (1998a) The coumarin 7-hydroxylation microassay in living hepatic cells in culture. *Alternatives to Laboratory Animals* **26:**213-223.

Donato MT, Gómez-Lechón MJ, Jover R, Nakamura T and Castell JV (1998b) Human hepatocyte growth factor down-regulates the expression of cytochrome P450 isozymes in human hepatocytes in primary culture. *J Pharmacol Exp Ther* **284:**760-767.

Doran E, Whittington FW, Wood JD, and J. D. McGivan JD (2002) Cytochrome P450IIe1 (CYP2E1) is induced by skatole and this induction is blocked by androstenone in isolated pig hepatocytes. *Chem-Biol Interact* **140**:81-92.

Ferrara R, Zanovello A, Bortolato S, White IN and Manno M (2001) Bioactivation and cytotoxicity of 1,1-dichloro-2,2,2,trifluoroethane (HCFC-123) in isolated rat hepatocytes. *Pharmacol Toxicol* **88:**192-197.

Friis C (1992) Distribution, metabolic fate and elimination of skatole in the pig. In: Measurement and Prevention of Boar Taint in Entire Male Pigs, In: Proc. EAAP Working Group: *Production and Utilization of Meat from Entire Male Pigs,* Roskilde, Denmark.

Friis C (1995) Is boar-taint related to sex differences or polymorphism of skatole metabolism, In: Proc. EAAP Working Group: *Production and Utilization of Meat from Entire Male Pigs*, United Kingdom.

Huijzer JC, Adams JD, Jaw J and Yost GS (1989) Inhibition of 3-methylindole bioactivation by the cytochrome P-450 suicide substrates 1-aminobenzotriazole and alpha-methylbenzylaminobenzotriazole. *Drug Metab Dispos* **17:**37-42.

Jiang Y, Kuo CL, Pernecky SJ and Piper WN (1998) The detection of cytochrome P450 2E1 and its catalytic activity in rat testis. *Biochem Biophys Res Commun* **246:**578-583.

Kharasch ED, Hankins DC, Baxter PJ and Thummel KE (1998) Single-dose disulfiram does not inhibit CYP2A6 activity. *Clin Pharmacol Ther* **64**:39-45.

Lanza DL and Yost GS (2001) Selective dehydrogenation/oxygenation of 3-methylindole by cytochrome P450 enzymes. *Drug Metab Dispos* **29:**950-953.

Lin Z, Lou Y, Squires E.J. (2004) Molecular cloning and functional analysis of porcine SULT1A1 gene and its variant: a single mutation SULT1A1 causes a significant decrease in sulfation activity*. Mamm Genome* **15**:218-26.

Loose DS, Kan PB, Hirst MA, Marcus RA and Feldman D (1983) Ketoconazole blocks adrenal steroidogenesis by inhibiting cytochrome P450-dependant enzymes. *J Clin Investig* **71:**1495-1499.

Meschter CL, Mico BA, Mortillo M, Feldman D, Garland WA, Riley JA and Kaufman LS (1994) A 13-week toxicologic and pathologic evaluation of prolonged cytochromes P450 inhibition by 1-aminobenzotriazole in male rats. *Fundam Appl Toxicol* **22:**369-381.

Pass GJ and Mclean S (2002) Inhibition of the microsomal metabolism of 1,8-cineole in the common brushtail possum (*Trichosurus vulpecula*) by terpenes and other chemicals. *Xenobiotica* **32:**1109-1126.

Peehl DM, Seto E, Hsu J and Feldman D (2002) Preclinical activity of ketoconazole in combination with calcitriol or the vitamin D analogue Eb 1089 in prostate cancer cells. *J Urol* **168:**1583-1588.

Ruangyuttikarn W, Appleton ML and Yost GS (1991) Metabolism of 3-methylindole in human tissues. *Drug Metab Dispos* **19:**977-984.

Schwarzenberger F, Toole GS, Christie HL and Raeside JI (1993) Plasma levels of several androgens and estrogens from birth to puberty in male domestic pigs. *Acta Endocrinol* **128**:173-177.

Sellers EM, Kaplan HL and Tyndale RF (2000) Inhibition of cytochrome P450 2A6 increases nicotine's oral bioavailability and decreases smoking. *Clin Pharmacol Ther* **68:**35-43.

Sinclair PA, Hancock S, Gilmore WJ, Squires EJ (2005) Metabolism of the 16-androstene steroids in primary cultured porcine hepatocytes. *J Steroid Biochem Mol Biol*. **96**:79-87.

Skaanild MT and Friis C (2005) Porcine CYP2A polymorphisms and activity. *Basic Clin. Pharmacol. Toxicol.* **97**:115-121.

Smets FN, Chen Y, Wang L and Soriano HE (2002) Loss of cell anchorage triggers apoptosis (anoikis) in primary mouse hepatocytes. *Molecular Genetics and Metabolism* **75:**344-352.

Smith DJ, Appleton ML, Carlson JR and Yost GS (1996) Identification of beta-glucuronidase-resistant diastereomeric glucuronides of 3-hydroxy-3-methyloxindole formed during 3-methylindole metabolism in goats. *Drug Metab Dispos* **24:**119-125.

Spracklin DK, Hankins DC, Fisher JM, Thummel KE and Kharasch ED (1997) Cytochrome P450 2E1 is the principal catalyst of human oxidative halothane metabolism *in vitro*. *J Pharmacol Exp Ther* **281:**400-411.

Squires EJ and Bonneau M (2004) Boar taint, in Control Encyclopedia of Meat Sciences (Jensen WK, Devine C and Dikeman M eds). Article 9, Academic Press, Oxford.

Squires EJ and Lundstrom K (1997) Relationship between cytochrome P4502E1 in liver and levels of skatole and its metabolites in intact male pigs. *J Anim Sci* **75:**2506-2511.

Thornton-Manning J, Appleton ML, Gonzalez FJ and Yost GS (1996) Metabolism of 3-methylindole by Vaccinia-expressed P450 enzymes: Correlation of 3-methyleneindolenine formation and protein-binding. *J Pharmacol Exp Ther* **276:**21-29.

Turini A, Amato G, Longo V and Gervasi PG (1998) Oxidation of methyl- and ethyl- tertiary-butyl ethers in rat liver microsomes: role of the cytochrome P450 isoforms. *Arch Toxicol* **72**.

Yost GS (1989) Mechanisms of 3-methylindole pneumotoxicity. *Chem Res Toxicol* **2:**273-279.

Zhang W, Kilicarslan T, Tyndale RF and Sellers EM (2001) Evaluation of methoxsalen, tranylcypromine, and tryptamine as specific and selective CYP2A6 inhibitors in vitro. *Drug Metab Dispos* **26:**897-902.

Footnotes

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Legends for figures

Figure I: Determination of basal hepatic microsomal P450 activities in different male pigs. A: PNP hydroxylase activity following treatment of microsomes with 200 µM PNP, B: COH activity following treatment of microsomes with 200 µM coumarin. N = 13 livers from 3-week old male pigs and mature boars, n = 5 for castrated males, with all samples analysed in duplicate on HPLC. Values are presented as means ± SE with groups not sharing a letter significantly different ($P<0.001$).

Figure II: Inhibitions of COH (CYP2A) and PNP hydroxylase (CYP2E1) activity in primary cultured porcine hepatocytes. PNP hydroxylase activity was measured by treatment of cells with 500 µM PNP. COH activity was measured by treatment of cells with 100 µM coumarin. Each data point represents 3 cell cultures analysed in duplicate on HPLC. A: Treatment of 4MP ranged from 0.1 – 100 µM; B: Treatment of 8MOP ranged from 0.1 – 500 µM; C: Treatment of DDTC ranged from 1 – 1000 µM. Values are plotted as means ± SE.

Figure III: Inhibition of PNP hydroxylase (CYP2E1) and COH (CYP2A) activity and the production of major 3MI metabolites (HMOI and 3MOI) in porcine hepatocytes treated with P450 inhibitors, 50 µM 4MP, 200 µM 8-MOP, or 250 µM DDTC. 3MI metabolites were measured by HPLC following treatment with 500 µM 3MI. A: CYP2E1 and CYP2A activity, B. production of HMOI and 3MOI. Each data point represents results obtained from 3 aliquots of cells from 3 different pig livers and all samples analysed in duplicate on HPLC. The results were divided by the control average from 5 aliquots of cells for each liver and reported as percentage of control. Values are presented as means ± SE with significant differences within P450 isoform or 3MI metabolite indicated by different letters. Capital letters refer to CYP2E1 and HMOI while lower case letters refer to CYP2A and 3MOI.

Figure IV: The effect of general P450 inhibitors KCZ and ABT on the activity of PNP hydroxylase (CYP2E1) and COH (CYP2A) and the production of 3MI metabolites HMOI and 3MOI in porcine hepatocytes. A: CYP2E1 and CYP2A activity, B. production of HMOI and 3MOI. Each data point represents results obtained from 3 aliquots of cells from 2 different pig livers and all samples analysed in duplicate on HPLC. The results were divided by the control average from 5 aliquots of cells for each liver and reported as percentage of control. Values are presented as means ± SE with significant differences within P450 isoform or 3MI metabolite indicated by different letters. Capital letters refer to CYP2E1 and HMOI while lower case letters refer to CYP2A and 3MOI. The HMOI peak was not resolved from ABT so these results were not available (N/A)

Table I: 3MI and metabolite standards used to identify peaks at specific retention times on HPLC.

Standard	**Detector**	**Concentration of standard (μM)**	**Present in cultured cells**	**Typical retention time (minutes)**
3-methyloxindole	UV	15	Yes	15.7
3-hydroxy-3-methyloxindole	UV	45	Yes	9.8
2-aminoacetophenone	UV	45	Yes	18.8
5-hydroxy-3-methylindole	FL	15	No	13.7
6-hydroxy-3-methylindole	FL	15	No	16.6
Indole-3-carbinol	FL	15	No	13.0
6-hydroxy-3-methylindole sulfate	FL	15	No	15.9
3-methylindole	FL	15	Yes	25.5

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Figure I

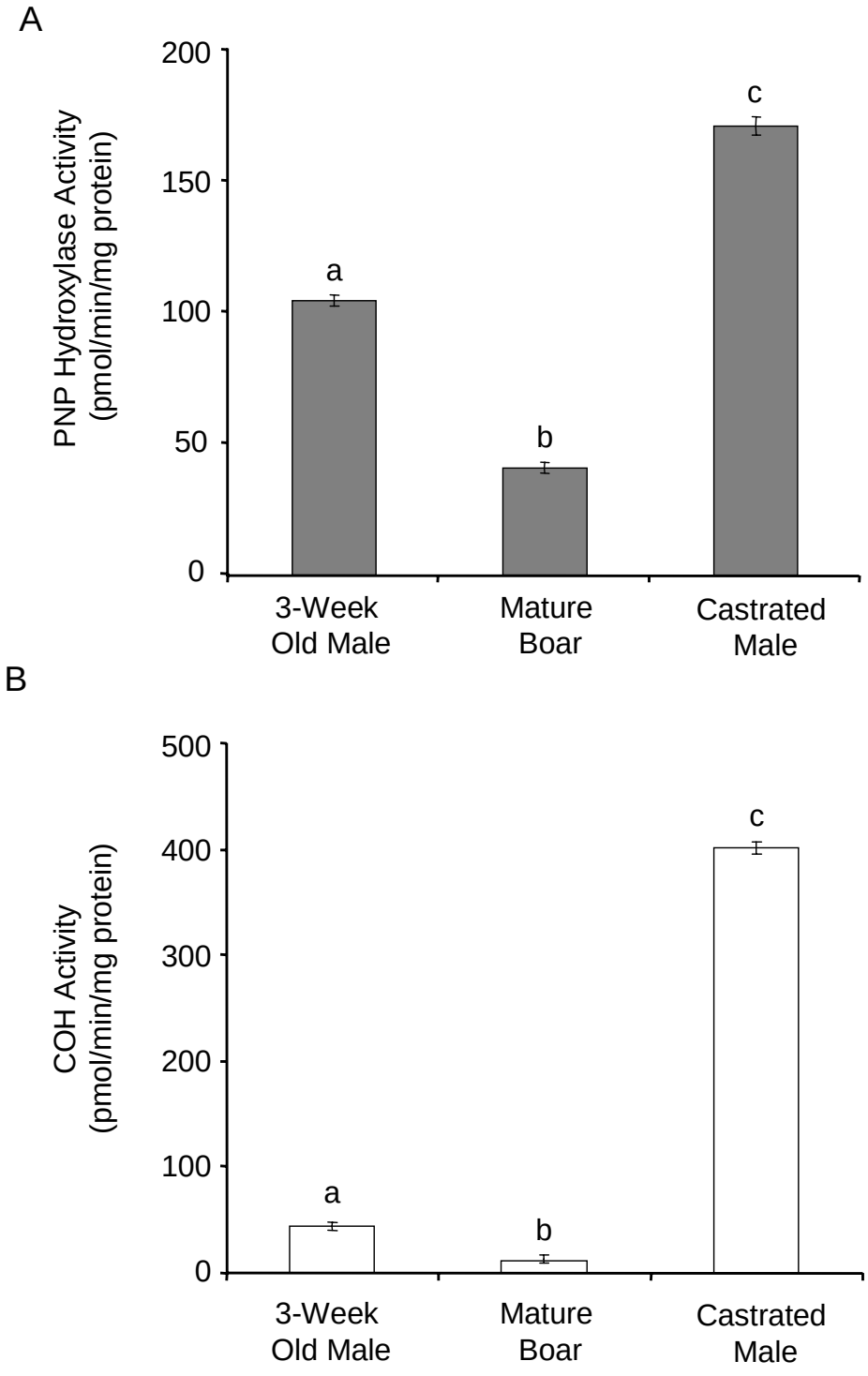

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Figure II

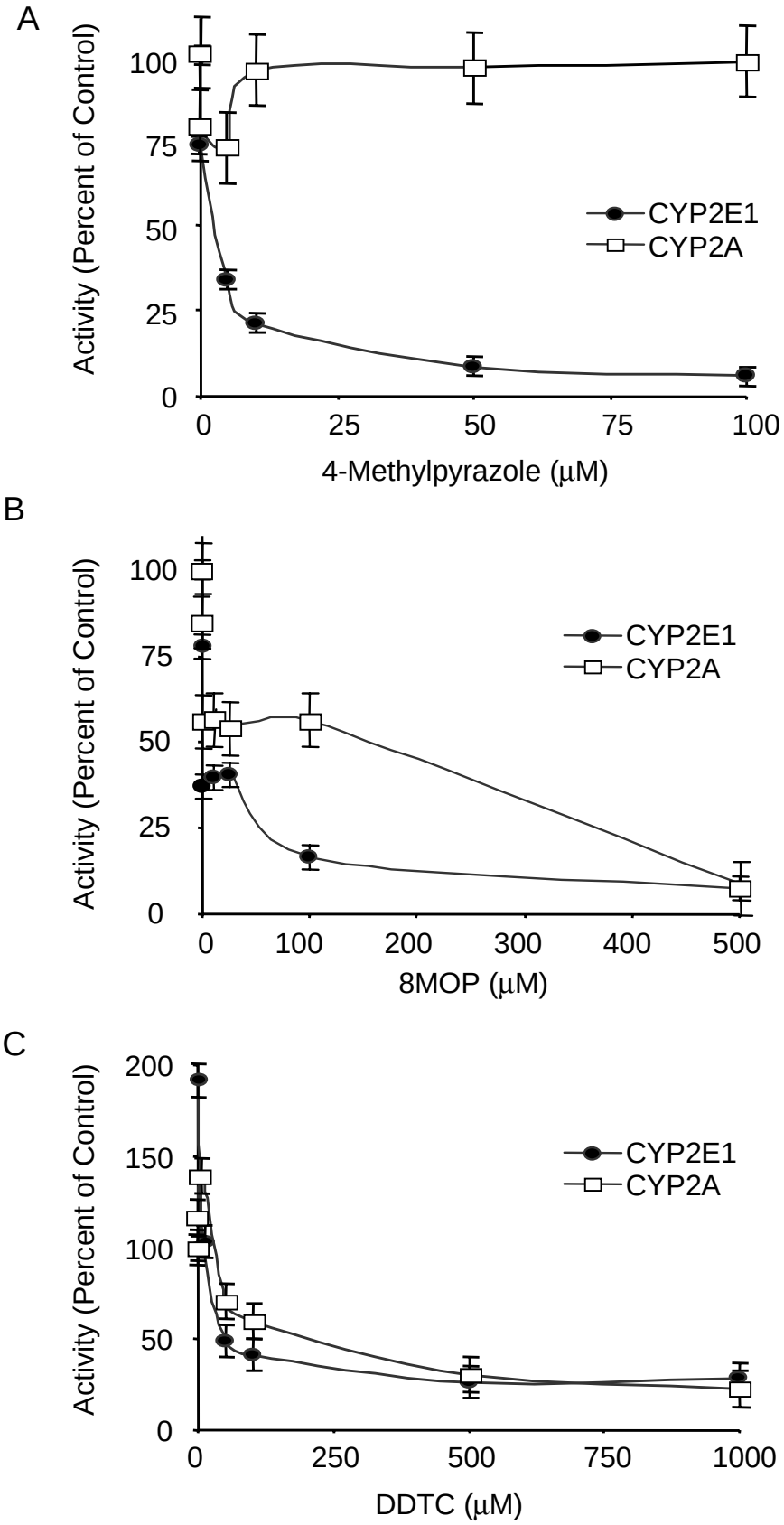

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Figure III

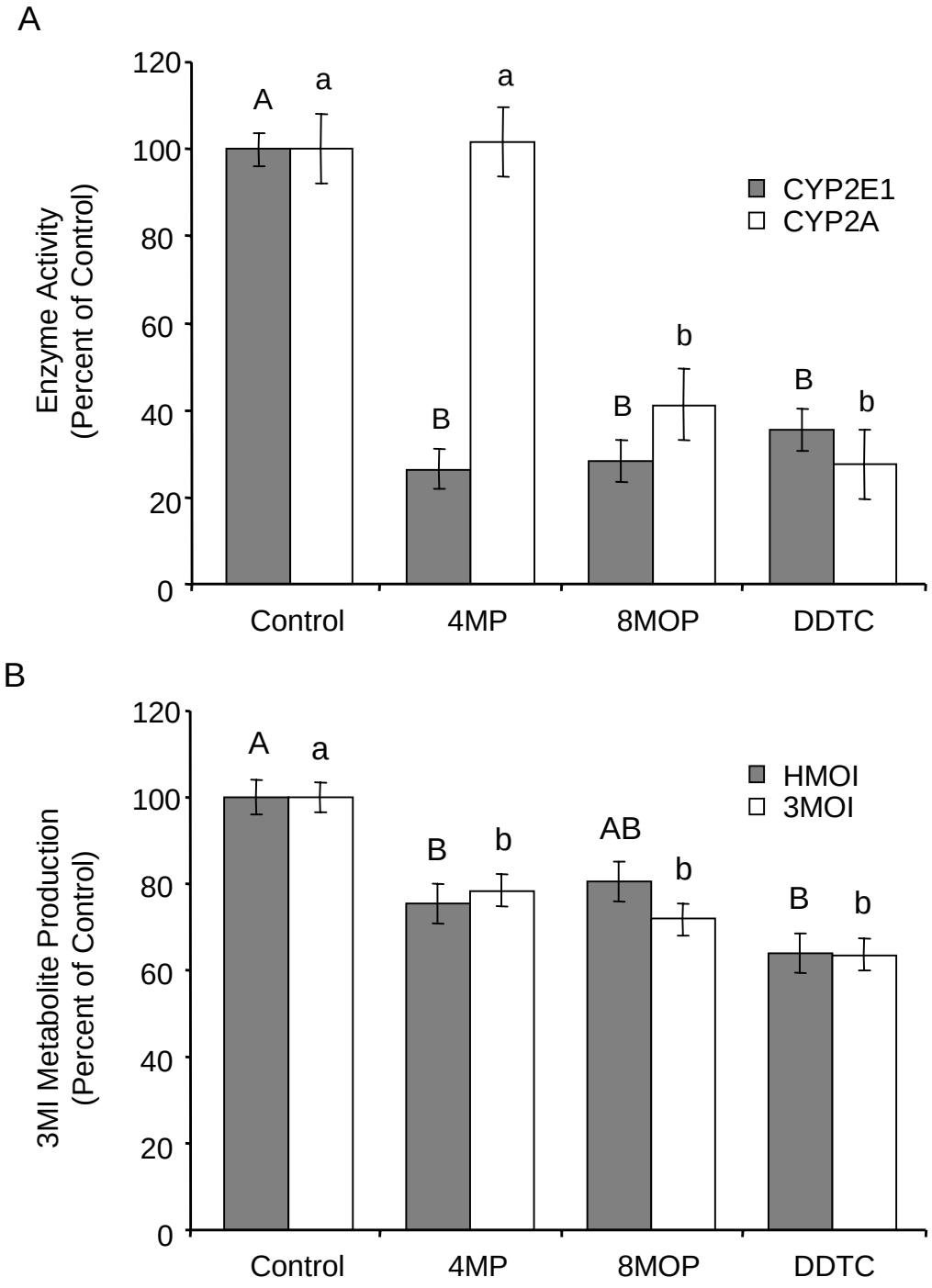

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Figure IV