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# Effect of testicular steroids on catalytic activities of cytochrome P450 enzymes in porcine liver microsomes

G. Zamaratskaia [a,*], W.J. Gilmore [b], K. Lundström [a], E.J. Squires [b]

[a] Department of Food Science, Swedish University of Agricultural Sciences, Box 7051, SE-750 07 Uppsala, Sweden
[b] Department of Animal and Poultry Science, University of Guelph, Guelph, Ont., Canada N1G 2W1

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**Abstract**

The testicular steroids androstenone (A), 17β-oestradiol (E2) and testosterone (T) were tested for their ability to alter CYP2E1 and CYP2A activity in porcine liver microsomes from male and female pigs. This is the first in vitro study indicating that sex steroids have a potential to modify microsomal CYP2E1 activity, the main skatole-metabolising enzyme. A and E2 exerted an inhibitory effect on CYP2E1 mediated hydroxylation of *p*-nitrophenol to *p*-nitrocatechol although the mechanism of this inhibition differed for these steroids. The inhibitory effect of A on CYP2E1, as determined by kinetic analysis, might be due to the competitive binding of A and *p*-nitrophenol to the same site of CYP2E1. Including E2 into the incubations resulted in decreased activities of CYP2E1 in male microsomes through a mixed mode of inhibition. Including pre-incubation steps eliminated this inhibition in male microsomes, and resulted in increased CYP2E1 activities in the microsomes from female pigs. Testosterone was ineffective as an inhibitor of either CYP2E1 or CYP2A activities. Overall, our findings indicate that A and E2 have the potential to modify the catalytic activities of porcine CYP2E1 in vitro. However, the significance of this modification for skatole metabolism in vivo is questionable.


*Keywords:* Androstenone; 17β-Oestradiol; Testosterone; Porcine microsomes; CYP2E1; CYP2A

## 1. Introduction

Skatole (3-methylindole) is a major contributor to a faecal-like odour in meat from entire male pigs. Skatole is produced in the large intestine of the pig and biotransformed in the liver via a two step process, phase I and phase II. The phase I step is responsible for the activation of the parent compound which typically introduces a hydroxyl group. This newly formed group then allows phase II enzymes to conjugate a variety of substrates. In general, the significance of this metabolism is to convert small lipophilic molecules into larger more water soluble compounds that are readily excreted. For skatole, conversion into more polar compounds is beneficial for its elimination. The majority of phase I skatole metabolism occurs through the cytochrome P450 system, a superfamily of heme-containing isoenzymes located within the endoplasmic reticulum in hepatocytes. Currently, cytochrome P450 (CYP) isoforms CYP2E1 and CYP2A are thought to be primarily responsible for the oxidative metabolism of skatole (Babol et al., 1998; Diaz and Squires, 2000a,b). Low activities of these enzymes in the liver may lead to decreased skatole metabolism and higher skatole accumulation in adipose tissue (Diaz and Squires, 2000b; Zamaratskaia et al., 2005a).

High skatole concentrations occur mainly in entire (uncastrated) male pigs, and not in either castrated males or female pigs. The mechanism responsible for the sex-related differences in skatole concentrations is not yet completely understood; however, it appears that liver metabolism extensively affects skatole accumulation in fat (Diaz and Squires, 2000b; Zamaratskaia et al., 2006). Entire male pigs expressed lower levels of hepatic CYP2E1 compared to

* Corresponding author. Tel.: +46 18 67 20 05; fax: +46 18 67 29 95.
*E-mail address:* Galia.Zamaratskaia@lmv.slu.se (G. Zamaratskaia).

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that of castrates (Whittington et al., 2004), and had lower CYP2E1 activity compared to that of female pigs (Zamaratskaia et al., 2006). Interestingly, the differences in CYP2E1 activity between entire male and female pigs were observed only in mature pigs at 115 kg live weight, whereas the activities at 90 kg (pre-puberty) did not differ between the genders (Zamaratskaia et al., 2006).

Decreased metabolism and hepatic clearance of skatole in mature entire male pigs can be due to the presence of testicular steroids at puberty. In contrast to males of other species, entire male pigs produce high amounts of oestrogens (Claus and Hoffmann, 1980; Raeside and Renaud, 1983), which are positively correlated to skatole levels (Zamaratskaia et al., 2005b,c). However, the mechanism by which high levels of oestrogens might affect skatole accumulation remains unknown.

The hepatic metabolism of testicular steroids, similar to skatole, is dependent on cytochrome P450s. Inhibition or decreased metabolism of skatole can result from competition between skatole and steroids for the enzyme's binding sites, modification of the enzyme by a reactive metabolite, or formation of a catalytically inactive complex between the enzyme and a steroid.

Androstenone, another boar taint compound, was shown to be involved in skatole metabolism either by direct enzyme inhibition (Babol et al., 1999) or by the inhibition of skatole-induced CYP2E1 expression (Doran et al., 2002; Whittington et al., 2004). Recently, Tambyrajah et al. (2004) also showed that androstenone decreases CYP2E1 promoter activity by inhibiting the binding of a transcription factor. Additionally, there is evidence that CYP2A is also strongly regulated by testicular steroids at the transcriptional level (Gillberg et al., 2006).

The aim of the present study was to further investigate the role of testicular steroids in hepatic skatole metabolism. For this purpose, the possibility of inhibition of CYP2E1 and CYP2A activities by androstenone (A), 17β-oestradiol (E2) and testosterone (T) was investigated by microsomal studies.

## 2. Material and methods

### 2.1. Microsomal incubation for analysis of CYP2A and CYP2E1 activities

Liver samples from entire male and female pigs of a crossbred (Swedish Yorkshire dams × Landrace sires) at live weight of approximately 90 kg were taken at slaughter, frozen in liquid nitrogen and stored in −80 °C for later enzymatic assays. The microsomal fraction was prepared as described previously (Diaz and Squires, 2000b).

Hydroxylation of *p*-nitrophenol to *p*-nitrocatechol was measured to determine the catalytic activity of CYP2E1 (Zamaratskaia et al., 2006). Incubations in a final volume of 0.25 ml contained 0.5 mg of microsomal protein, 0.2 mM of *p*-nitrophenol and 1.0 mM NADPH in incubation buffer (0.1 M potassium phosphate, pH 6.8). Incubations were performed in glass test tubes in a water bath at 37 °C, for 15 min. Reactions were terminated with 0.02 ml of 40% trichloroacetic acid, followed by centrifugation at 13000*g* for 15 min. Measurements of the product *p*-nitrocatechol in supernatants were performed on HPLC with UV detector set at 345 nm. HPLC analyses were performed on a system consisted of a pumping system (L-6200A), autosampler (AS 2000), UV detector (SP Spectra 100) and D-6000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan).

The catalytic activity of CYP2A was measured as the rate in formation of 7-hydroxycoumarin from coumarin (Zamaratskaia et al., 2006). Incubations in a final volume of 0.4 ml contained 0.5 mg of microsomal protein, 0.2 mM of coumarin and 1.6 mM NADPH in incubation buffer (50 mM Tris, 5 mM $MgCl_2$, pH 7.4). Incubations were performed in glass test tubes in a water bath at 37 °C, for 15 min. Reactions were terminated with 0.1 ml of 20% trichloroacetic acid, followed by centrifugation at 13000*g* for 5 min. The concentrations of the product 7-hydroxycoumarin in supernatants were measured by HPLC with fluorescence detector (excitation and emission wavelengths of 338 and 458 nm, respectively) (Souček, 1999). HPLC analyses were performed on a system consisted of a pumping system (L-7100), autosampler (L-7200), fluorescence detector (L-7485) and D-7000 HPLC Manager software (Merck, Hitachi, Tokyo, Japan). A reversed-phase Li-Chrospher RP-18 column (5 μm) equipped with a guard column was used in all HPLC analyses.

The rate of metabolite formation was linear with the times of incubation and protein concentrations for both assays. The formation of metabolites were determined by interpolation of a standard curves made with authentic standards.

### 2.2. Inhibition studies

Inhibition studies were performed on individual microsomes from 5 male and 5 female pigs using two approaches. First, to evaluate the possibility of a direct inhibitory effect of testicular steroids on the catalytic activities of CYP2E1 and CYP2A, the assays were conducted in the presence of varying concentrations of androstenone (A), 17β-oestradiol (E2) and testosterone (T) (0.1, 1, 10 and 50 μM). Steroids were dissolved in methanol and the final content of methanol was 0.1% in the reaction volumes of 0.25 and 0.4 ml for CYP2E1 and CYP2A, respectively. The same amount of methanol was added in the control reactions. The percent of control enzymatic activity was calculated for each steroid.

Second, the possibility of NADPH-dependent metabolite formation for steroids was investigated by a pre-incubation in the presence of NADPH. Microsomes were pre-incubated with steroids and NADPH for 15 min; following pre-incubation, probe substrates, coumarin for CYP2A and *p*-nitrophenol for CYP2E1, were added to the incubations, the reactions were allowed to proceed and the activities were measured as described above. Pre-incubation time of 15 min has been shown to elicit the highest ability, if any, of steroids to modify enzyme activity.

### 2.3. Inhibition kinetics

The enzyme kinetic studies were performed by using a pool of microsomes from six male pigs for the determination of inhibition mode by E2, and a pool of microsomes from three male and three female pigs for the determination of inhibition mode by A. Microsome pools were formed based on the results from the inhibition study with individual microsomes. Addition of A affected CYP2E1 and CYP2A activities in both sexes in a similar manner, while E2 decreased enzyme activities only in male microsomes. The apparent kinetic constants (Michaelis constant $K_m$ and maximal rate of product formation $V_{max}$) were estimated by nonlinear regression analysis (Enzyme Kinetics Pro, version 2.36, SynexChem, Fairfield, CA) using the Michaelis–Menten model: $V = V_{max}[S]/(K_m + [S])$. The initial estimates of $K_i$ values (equilibrium dissociation constant for the enzyme–inhibitor complex) were estimated from the Lineweaver–Burk plot for the substrate range from 0.0125 to 0.1 mM for coumarin and from 0.02 to 0.1 mM for *p*-nitrophenol. The analysis was performed using the single inhibitor concentration of 1 μM at which the initial inhibitions were observed. The inhibition modes were determined by fitting the enzyme activity–substrate concentration data to the equations