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A novel gender-related difference in the constitutive expression of hepatic cytochrome P4501A subfamily enzymes in Meishan pigs

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ABSTRACT

Constitutive expression levels of hepatic CYP1A subfamily enzymes, CYP1A1 and CYP1A2, in male and female Meishan pigs were examined at levels of the mRNA, protein, and enzyme activity. In mature (5-month-old) pigs, levels of hepatic CYP1A1 and CYP1A2 mRNAs, as determined by RT-PCR, were much higher in females than in males, but those of castrated male pigs were equivalent to female pigs. The gender-related differences in the levels of CYP1A mRNAs closely correlated with those of the corresponding apoproteins determined by Western blotting. Hepatic enzyme activities not only for the *O*-dealkylation of ethoxyresorufin and methoxyresorufin (typical substrates for CYP1A1 and CYP1A2, respectively) but also for the mutagenic activation of benzo[*a*]pyrene and 2-amino-6-methyl-dipyrido[1,2-*a*; 3′,2′-*d*]imidazole (typical substrates for CYP1A1 and CYP1A2, respectively) were also much greater in female and castrated male pigs than in male pigs. In immature (1-month-old) pigs, no such gender-related differences were observed, and their gene expression levels of the CYP1A subfamily enzymes were almost the same as those of mature female pigs. Furthermore, treatment of immature pigs with testosterone resulted in a drastic decrease in the levels of the CYP1A1 and CYP1A2 mRNAs in both sexes. The present findings demonstrate a gender-related difference in the constitutive expression of hepatic CYP1A subfamily enzymes in Meishan pigs and further indicate that androgen down-regulates the constitutive gene expression of the enzymes.

1. Introduction

The liver plays an important role in the metabolism of xenobiotics and steroid hormones and the biosynthesis/metabolism of cholesterol. The metabolism of xenobiotics and steroid hormones is mainly catalyzed by hepatic cytochrome P450 (P450), which consists of several subfamilies [1]. P450 subfamily enzymes have different substrate-specificities [2], and their expression levels are often influenced by a variety of host factors including sex, age, and physiological state.

Sexual dimorphism in the expression of several P450 subfamily enzymes, including CYP3A and CYP2C subfamily enzymes which are responsible for drug-metabolism, is well known [3–5]. Different secretion profile of growth hormone is

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Table 1 – Body weights of pigs used in the present study

Age (months)	Treatment	Number	Body weight (kg)		
			Male	Castrated male	Female
Experiment 1					
5	None	3	50.4 ± 4.2	55.3 ± 9.6	49.8 ± 8.7
1	None	3	5.5 ± 0.8	–	5.2 ± 0.5
Experiment 2					
1	None	3	6.5 ± 0.3	–	6.9 ± 1.1
1	Vehicle	1	6.8	–	5.0
1	TP	3	6.8 ± 0.2	–	7.4 ± 0.3

The values shown represent mean ± S.D. The vehicle was corn oil.

known to determine the sexually dimorphic constitutive expression of CYP2A, 2C, and 3A subfamily enzymes [5–7]. Likewise, androgen is also known to determine sexually dimorphic constitutive and/or xenobiotic-induced expressions of P450 subfamily enzymes, including Cyp2b [8], Cyp1a2 [9–12], and Cyp4b1 [13,14] in mice.

It is well known that CYP1A subfamily enzymes play an important role in the metabolism of many carcinogens and drugs. For example, CYP1A1 and CYP1A2 catalyze the metabolic activation of carcinogenic aryl hydrocarbons including benzo[*a*]pyrene [15] and carcinogenic aromatic amines including cooked food-derived heterocyclic amines [16,17]. Further, the activities of CYP1A1 and CYP1A2 in target tissues are one of the host factors that determine carcinogenic susceptibility of experimental animals toward the aryl hydrocarbons [15,18] and the aromatic amines [9–11,19], respectively.

Since many physiological characteristics of pigs are similar to those of humans, pharmacological and toxicological studies with pigs would contribute to our understanding of human responses to xenobiotics such as drugs and environmental chemicals. Recently, we have identified the cDNA sequences for several pig P450 subfamily enzymes including the CYP1A subfamily [20]. However, there is little information about the expression of hepatic P450 in pigs.

In the present study, we examined whether or not there is a gender-related difference in the constitutive expression of hepatic CYP1A subfamily enzymes, CYP1A1 and CYP1A2, in Meishan pigs. Results indicate that there is a gender-related difference in the constitutive expression of CYP1A isoforms and further confirm that pig CYP1A1 and CYP1A2 enzymes have a clear ability to activate benzo[*a*]pyrene and 2-amino-6-methyl-dipyrido[1,2-*a*; 3′,2′-*d*]imidazole to mutagenic forms, respectively.

2. Materials and methods

2.1. Animals

Meishan pigs were bred and kept at the National Institute of Livestock and Grassland Science, Tsukuba, Japan. Pigs were fed a commercial grain diet and provided with water *ad libitum*. Pigs were killed at the age of 5 months or 1 month. Castration was performed at the age of 1 month, and castrated pigs were killed at 5 months of age. After each animal in experimental groups was killed, a portion of the liver was quickly removed, frozen in liquid nitrogen, and kept at −80 °C for subsequent analysis. The mean body weights of the Meishan pigs used in the present study are shown in Table 1.

2.2. Treatment with testosterone

Testosterone propionate (TP; Sigma Chemical Co., St. Louis, MO) was administered to immature (1-month-old) male and female pigs. In brief, the testosterone propionate (50 mg/ml) dissolved in corn oil was intramuscularly injected, at a dose of 10 mg/kg, to the rear leg of the pig five times with a 1-day intervals. Pigs were killed 24 h after the last injection.

2.3. Gene expression of hepatic CYP1A subfamily enzymes

Total RNA was prepared from individual livers using TRIzol Reagent (Invitrogen Corp., Carlsbad, CA, USA) and used to determine the level of the gene expression of CYP1A isoforms, CYP1A1 and CYP1A2. Briefly, a portion (4 μg) of total RNA was converted to cDNA in 20 μl of reverse transcription (RT)-reaction mixture using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen) and oligo $d(T)_{12–18}$ according to the manufacturer's instructions. PCR was performed in a 25 μl reaction mixture containing 0.5 μl of the RT-reaction mixture, 200 μM of dNTP, 1 U of AmpliTaq Gold, and 400 nM of each primer (forward and reverse). Primer sequences are shown in Table 2. A primer pair specific for CYP1A2 was designed on basis of the cDNA sequences of CYP1A1 (GenBank accession no. AB052254) and CYP1A2 (GenBank accession no. BP442953). Primers for glyceraldehydes 3-phosphate dehydrogenase (G3PDH), which was used as an internal standard, were obtained from Toyobo (Tokyo, Japan). The amplification protocol was as follows; preactivation of AmpliTaq Gold for 10 min at 95 °C and then 23–25 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 55 °C, and extension for 30 s at 72 °C. The PCR products were electrophoresed in 2% agarose gel and visualized using ethidium bromide. Gene products were semi-quantified by measuring the intensity of bands with a Luminous Imager (Aisin Cosmos R&D Co. Ltd., Kariya, Japan).

2.4. Preparation of hepatic microsomes

Microsomal fractions were prepared from liver homogenates by differential centrifugations as described previously [10].

Table 2 – Primer pairs used in the present study

Gene	Forward primer	Reverse primer	Length (bp)	Accession no.
CYP1A1	atcctggagctcttccgac	ggtatgatccctcaggcttg	584	AB052254
CYP1A2	gtgaggagatgttcagcatcgtgaag	cttctgtatctcaggatatgtcaca	386	BP442953

The amount of microsomal protein was determined by the method of Lowry et al. [21].

2.5. Western blot analysis of CYP1A proteins

Western blot analysis was performed according to the method of Degawa et al. [11] with a slight modification. In brief, each hepatic microsomal preparation (50 μg protein/lane) was separated by 9% SDS-polyacrylamide gel electrophoresis. The separated proteins were transferred from the gel to a nitrocellulose membrane, Hybond-ECL (GE Healthcare Bio-Sciences Corp., Piscataway, NJ, USA), and then immunostained by reaction with the primary antibody followed by reaction with horseradish peroxidase (HRP)-conjugated secondary antibody. A goat anti-rat CYP1A2 serum (''Antiserum for human CYP1A1/1A2'': Daiichi Pure Chemicals, Tokyo, Japan), which is cross-reactive with human CYP1A1/1A2, was used as the first antibody. An HRP-conjugated rabbit anti-goat IgG (Sigma) was used as the second antibody, and 0.05% 3,3′-diaminobenzidine tetrachloride (Wako Pure Chemical Industries Ltd., Osaka, Japan) was used as the enzyme substrate.

2.6. CYP1A subfamily enzyme activities

Hepatic microsomal activities for the ethoxyresorufin *O*-deethylation (EROD) and methoxyresorufin *O*-demethylation (MROD), which are mainly catalyzed by CYP1A1 and CYP1A2, respectively [22,23], were determined by the method of Degawa et al. [24]. Briefly, a reaction medium (250 μl of 0.1 M sodium phosphate buffer, pH 7.4) containing hepatic microsomes (150 μg) and an NADPH-generating system was pre-incubated at 37 °C for 10 min, and the reaction was started by the addition of 5 μl of methoxyresorufin or ethoxyresorufin (250 μM) dissolved in dimethylsulfoxide. After incubation at 37 °C for 10 min, 250 μl of cold ethanol was added to stop the reaction. The reaction mixture was centrifuged for 10 min at 2500 × *g*, and the amount of resorufin formed in the resulting supernatant was measured using a Wallac 1420 ARVOsx Mutilabel Counter (PerkinElmer Life Sciences, Wellesley, MA) at an excitation wavelength of 550 nm and an emission wavelength of 590 nm. In addition, hepatic microsomal EROD and MROD activities increased in a reaction time-dependent manner at least until 10 min.

Since benzo[*a*]pyrene (B[*a*]P) and 2-amino-6-methyl-dipyrido[1,2-*a*;3′,2′-*d*]imidazole hydrochloride (Glu-P-1) are known to be selectively converted to mutagenic forms by CYP1A1 and CYP1A2, respectively [25], Ames' bacterial mutation assay using B[*a*]P and Glu-P-1 as premutagens was also performed according to the method of Degawa et al. [25]. In brief, 9000 × *g* supernatant (S9) was prepared from liver homogenates of pigs in each experimental group and used as an enzyme source. The S9 activities for mutagenic activations of B[*a*]P (100 nmol/plate) and Glu-P-1 (2 nmol/plate) toward *Salmonella typhimurium* TA98 were examined.

2.7. Statistical analysis

Significant differences were evaluated using the Student's t-test.

3. Results

3.1. Gene expression levels of CYP1A subfamily enzymes in mature pigs

Five-month-old pigs were used as sexually mature pigs [26,27]. Constitutive gene expression levels of hepatic CYP1A subfamily enzymes, CYP1A1 and CYP1A2, were examined by RT-PCR in mature male and female pigs, and the results are shown in Fig. 1. The mRNAs of CYP1A1 and CYP1A2 were clearly detected in female pigs but faintly in male pigs (Fig. 1a). The gene expression levels of CYP1A subfamily enzymes were

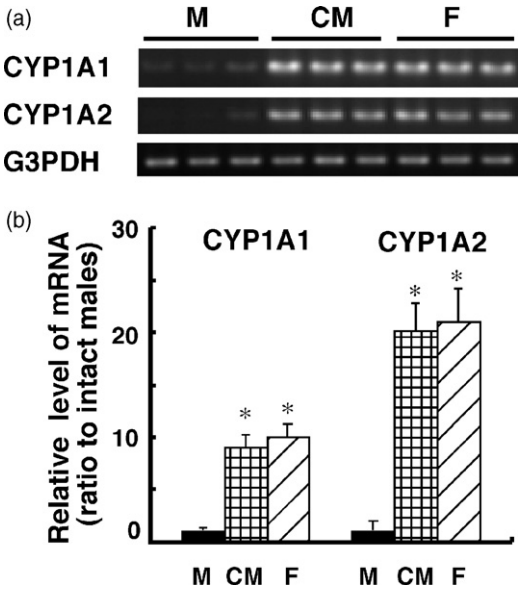

Fig. 1 – Hepatic gene expression of CYP1A1 and CYP1A2 in 5-month-old pigs. Total RNAs were prepared from individual livers of three pigs in each experimental group; intact male (M), castrated male (CM), and intact female (F). Four micrograms of total RNA was converted to cDNA, and the resulting cDNA was amplified by PCR. (a) Expression patterns for the *CYP1A1* and *CYP1A2* genes in individual pigs. Equal volumes of the PCR reaction mixtures were subjected to electrophoresis on a 2% agarose gel. (b) Gene expression levels of CYP1A1 and CYP1A2 were normalized to that of G3PDH, an internal standard, and shown as ratios to the corresponding intact male pigs. Each column indicates the mean in each experimental group, and bars represent the standard deviation of the mean ($n = 3$). Significantly different from intact male pigs: $^{*}P < 0.01$.

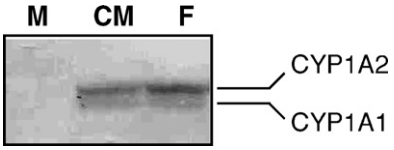

Fig. 2 – Western blot analysis of hepatic CYP1A apoproteins in 5-month-old pigs. Hepatic microsomal fractions were prepared from the livers of intact male (M), castrated male (CM), and female (F) pigs. The pooled microsomes (50 μg/lane) from three pigs per experimental group were used for Western blot analysis.

much higher in female pigs than in male pigs (Fig. 1b). In addition, castration of male pigs led to a female-like gene expression pattern of CYP1A subfamily enzymes (Fig. 1a and b).

3.2. *Western blot analysis of hepatic CYP1A apoproteins in mature pigs*

Levels of CYP1A1 and CYP1A2 apoproteins were comparatively examined among the mature male, castrated male, and female pigs. The bands corresponding to CYP1A1 and CYP1A2, which were determined on the basis of data described by Myers et al. [28], were clearly detected in castrated male pigs and mature female pigs, but barely detectable in intact male pigs (Fig. 2).

3.3. *Gender-related differences in CYP1A subfamily enzyme activity*

CYP1A subfamily enzyme activities were examined in mature male, castrated male, and female pigs. The enzyme assays were performed using ethoxyresorufin and methoxyresorufin as typical substrates for CYP1A1 and CYP1A2, respectively. Hepatic microsomal activities for ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) were 10-fold and 20-fold higher, respectively, in female pigs than in male pigs (Fig. 3a). In castrated male pigs, the activities for EROD and MROD were equivalent to those of female pigs.

We also performed Ames' bacterial mutation assay using B[*a*]P and Glu-P-1, which are converted to mutagenic forms by the CYP1A1 and CYP1A2 [25], respectively. Hepatic S9 activities for mutagenic activations of B[*a*]P and Glu-P-1 toward *S. typhimurium* TA98 were much higher in female pigs than in male pigs (Fig. 3b). In castrated male pigs, the S9 activities were equivalent to those of female pigs.

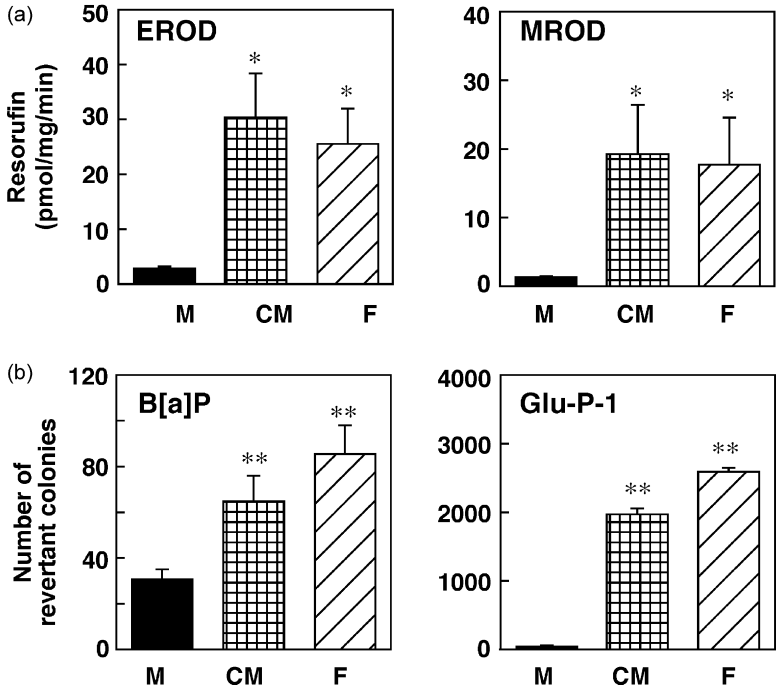

Fig. 3 – Activities of CYP1A enzymes in the 5-month-old pig liver. (a) Microsomal activities for EROD and MROD. Hepatic microsomal fractions were prepared from individual livers of three pigs in each experimental group; intact male (M), castrated male (CM) and intact female (F). The enzyme assays were performed as described in Section 2. Each column represents the mean in each experimental group, and bars represent the standard deviation of the mean ($n = 3$). (b) S9 activities for mutagenic activation of B[*a*]P and Glu-P-1 toward *S. typhimurium* TA98. CYP1A activities in hepatic S9 fractions (pooled from three pigs per experimental group) were determined by the Ames' test using B[*a*]P and Glu-P-1, which are converted to mutagenic forms by CYP1A1 and CYP1A2, respectively. Amounts of the S-9 used for B[*a*]P and Glu-P-1 were 1.5 mg protein/plate and 150 μg protein/plate, respectively. Mutagenicity toward *S. typhimurium* TA98 is shown as the number of revertant colonies/mg protein/plate. Each column represents the mean in each experimental group, and bars represent the standard error of the mean ($n = 4$). Significantly different from intact male pigs: $^{*}P < 0.05$, $^{**}P < 0.01$.

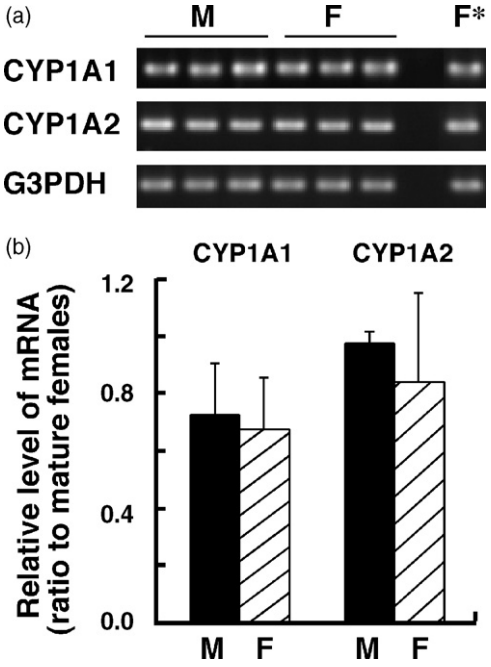

Fig. 4 – Hepatic gene expression levels of CYP1A1 and CYP1A2 in 1-month-old pigs. Total RNA was prepared from the liver of 1-month-old male (M) and female (F) pigs and from 5-month-old female pigs (F*; total RNA pooled from three pigs) as a positive control. Four micrograms of total RNA was converted to cDNA, and the resulting cDNA was amplified by PCR. (a) Equal volumes of the PCR reaction mixtures were subjected to electrophoresis on a 2% agarose gel. (b) Gene expression levels of CYP1A1 and CYP1A2 were normalized to that of G3PDH. Graphs show fold-change with respect to mature (5-month-old) female pigs. Each column represents the mean in each experimental group, and bars represent the standard deviation of the mean ($n = 3$).

3.4. *Gene expression levels of CYP1A subfamily enzymes in immature pigs*

One-month-old pigs were used as sexually immature pigs [26,27]. To clarify the relationship between constitutive gene expression of CYP1A subfamily enzymes and sexual-maturation, gene expression levels of the enzymes were compared between immature male and female pigs. RT-PCR analyses revealed that there was no gender-related difference in the constitutive gene expression of CYP1A subfamily enzymes, and expression levels of CYP1A1 and CYP1A2 in immature pigs were equivalent to those of mature female pigs (Fig. 4).

3.5. *Down-regulation of the gene expression of CYP1A subfamily enzymes by testosterone*

To determine whether or not androgen suppresses the constitutive gene expression of hepatic CYP1A1 and CYP1A2, testosterone propionate (TP) was administered to immature (1-month-old) male and female pigs. As shown in Fig. 5, treatment with TP led to a clear decrease in the gene expression of CYP1A1 and CYP1A2 in both sexes of pigs.

4. Discussion

In the present study, we found that there is a gender-related difference in the constitutive expression of hepatic CYP1A subfamily enzymes, CYP1A1 and CYP1A2, in mature (5-month-old) pigs. The mRNA and protein levels of CYP1A subfamily enzymes were much greater in female pigs than in males. Likewise, CYP1A enzyme activities were much higher in female than in male pigs.

Castration of male pigs led to a female-like expression pattern of the enzymes. Furthermore, no gender-related difference in the constitutive expression of CYP1A subfamily enzymes was observed in immature (1-month-old) pigs. These present findings proposed a hypothesis that androgen down-regulates the constitutive expression of CYP1A subfamily enzymes. To demonstrate this, we further examined the effects of androgen TP-administration on hepatic gene expression of CYP1A subfamily enzymes in immature male and female pigs and showed that the gene expression levels of CYP1A1 and CYP1A2 were drastically down-regulated by TP-treatment in both sexes of immature pigs. We previously reported the gender-related differences in the carcinogenic aromatic amine-mediated induction of Cyp1a2 in the mouse

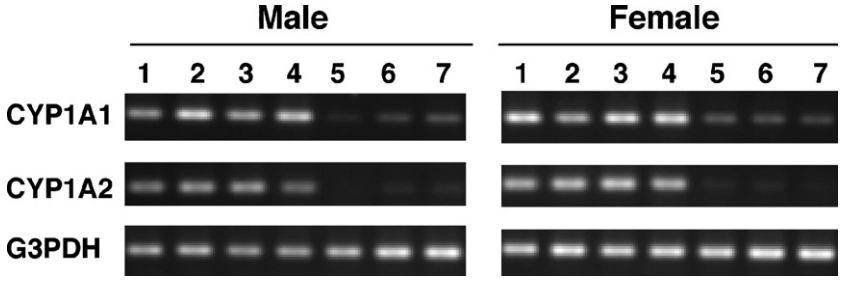

Fig. 5 – Effect of TP-treatment on hepatic gene expression of CYP1A1 and CYP1A2 in 1-month-old pigs. Testosterone propionate or vehicle (corn oil) was intramuscularly injected (10 mg/kg) five times with a 1-day interval to 1-month-old (immature) male and female Meishan pigs. Pigs were killed 24 h after the last treatment. Total RNA was prepared from individual livers of pigs, and 4 μg of total RNA was converted to cDNA, and the resulting cDNA was amplified by PCR. Equal volumes of the PCR products were subjected to electrophoresis on a 2% agarose gel, and bands were visualized by UV fluorescence of ethidium bromide. Lanes 1–3, untreated; lane 4, vehicle; lanes 5–7; TP-treatment.

liver [9–12] and the inhibitory effect of androgen on the gene expression of the enzyme [11,12]. These findings strongly suggest that androgen plays an important role in the constitutive and/or chemical-mediated gene expression of hepatic CYP1A subfamily enzymes, although the mechanism of androgen-mediated down-regulation of CYP1A subfamily genes remains unclear. In addition, the differential levels of constitutive and/or carcinogen-induced CYP1A subfamily enzymes in target tissues closely correlate with the susceptibility of experimental animals toward carcinogenic aryl hydrocarbons [15,18] and aromatic amines [9–11,19].

To date, sexual dimorphisms in the constitutive expression of the CYP2A, CYP3A, and CYP2C subfamily enzymes are reported to be due to differential secretion of growth hormone [5–7]. Hepatic CYP1A subfamily enzymes would not be regulated in this way. Concerning constitutive gene expression of hepatic CYP1A subfamily enzymes, a clear gender-related difference has not been found until our present findings.

In conclusion, we demonstrate for the first time the gender-related difference in constitutive expression of hepatic CYP1A subfamily enzymes in Meishan pigs and further show that the constitutive gene expression was down-regulated by androgen. Meishan pigs would be useful animals for further understanding of the mechanism underlying the gender-related difference in constitutive expression of CYP1A subfamily enzymes that play an important role in the metabolism of the endogenous and exogenous chemicals, including steroid hormones, drugs, and environmental carcinogens.

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