FULL PAPER *Toxicology*

Cytochrome P450 1A-Dependent Activities in Deer, Cattle and Horses

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ABSTRACT. The objective of this study was to investigate and characterize the metabolic activities of CYP1A in deer, cattle and horses in comparison to those of rats using ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) assays. We performed an inhibition study for these activities using anti-rat CYP1A1 antibody and identified that these activities were due to the CYP1A subfamily. Interspecies differences in the CYP1A-dependent activities were highly observed in this study. In particular, we found that the horse had the highest EROD and MROD activities among the examined animal species. In the kinetic analysis, the horses showed the highest Vmax and catalytic efficiency (Vmax/Km), followed by the cattle, deer and rats.

KEY WORDS: CYP1A1, cytochrome P450, equine, ethoxyresorufin O-deethylase, ungulates.

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The cytochrome P450 (CYP) superfamily comprises more than 5,000 genes encoding heme-thiolate enzymes that catalyze the oxidative metabolism of a vast array of organic compounds. The CYP1A subfamily has a broad affinity for polycyclic aromatic hydrocarbons, heterocyclic amines, endogenous substances and naturally occurring chemicals. Moreover, it plays important roles in both mediating and mitigating the biological effects of these chemicals and can determine susceptibility to toxicity or disease [4, 5, 7, 27].

In mammals, the CYP1A subfamily has two isoforms (CYP1A1 and CYP1A2), which have highly homologous amino acid sequences (more than 70% identity) [15, 20]. CYP1A1 and CYP1A2 are distinct but have overlapping substrate specificities. CYP1A1 primarily targets polycyclic aromatic hydrocarbons such as benzo[a]pyrene and 3-methylcholanthrene, whereas CYP1A2 catalyzes the metabolic activation of aryl and heterocyclic amines such as 2-acetylaminofluorene and the oxidative metabolism of drugs including phenacetin, warfarin, caffeine and theophylline [13].

Comparisons of the catalytic selectivity of individual CYP enzymes have been published for human and laboratory animals such as rodent species, dogs and monkeys [1, 6, 11, 24, 28]. However, only limited knowledge on hepatic CYP enzymes in other animal species, especially in ungulates like deer, cattle and horses, is currently available [17, 18, 25, 26]. These animals are exposed during their lifetimes to a host of xenobiotics such as drugs, growth promoters and environmental contaminants. Once absorbed, xenobiotics undergo a number of hepatic and extrahepatic enzymatic reactions referred to as biotransformations, which may be divided into phase I and phase II reactions [18]. Biotransformation reactions are able to influence in target species both the safety and effectiveness of drugs. At the same time, the reactions affect the sensitivity to several toxicants and the accumulation pattern of the chemical residues that animal products intended for human consumption may eventually accumulate [14].

In this study, we attempted to investigate more about the metabolic activity of CYP1A in cattle, deer and horses, in comparison to rats as a reference species, through measurement of the ethoxyresorufin O-deethylation (EROD) and methoxyresorufin O-demethylation (MROD) activities. Moreover, immunoinhibition of EROD and MROD activities was performed to prove that these activities were due to CYP1A, but not other CYPs, in this group of animals. In order to characterize the interspecies differences in CYP1A-dependent activities towards ethoxyresorufin, EROD kinetic parameters were clarified.

MATERIALS AND METHODS

Chemicals and reagents: All test substances and reagents used were of reagent grade including those described below. Resorufin, ethoxyresorufin and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). NADPH, glucose-6-phosphate (G-6-P) and glucose-6-phosphate dehydrogenase (G-6-PDH) were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan), polyclonal rabbit anti-rat CYP1A1 antibody was purchased from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan) and rabbit IgG was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The anti-rat CYP1A1 is reported to cross-react with CYP1A1 and CYP1A2 in rat microsomes. All other reagents were of analytical grade or the highest quality available and were purchased from Wako Pure Chemical Industries (Osaka, Japan).

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Animals: All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. Liver samples were collected from three adult female animals. Holstein cattle (*Bos taurus*) samples were purchased from a Hokkaido University cattle farm at the age of 4 to 5 years (4.67 ± 0.58 year old). Thoroughbred horse (*Equus caballus*) liver samples were kindly gifted to us by the JRA (Japan Racing Association, Japan) at the age of 4 to 6 years (5.33 ± 1.15 year old). Ezo shika deer (*Cervus hortulorum yesoensis*) were hunted in the wild (Hokkaido, Japan) during the winter season, and their ages were estimated based on tooth eruption and tooth wear patterns (2.00 ± 0.71 year old). The livers of the ungulates were excised immediately after slaughter or hunting and were flash-frozen in liquid nitrogen.

Nine week old Wistar female rats (SLC, Hamamatsu, Japan) were housed at 24 ± 1°C with a 12-hr light and 12-hr dark cycle and were given laboratory feed and water *ad libitum*. The rats were anaesthetized and killed with carbon dioxide. Livers were removed and perfused with cold 1.15% KCl to remove the blood.

Preparation of liver microsomes: Liver microsomes from these animals were prepared according to the methods described by Omura and Sato [21]. Briefly, livers were minced and homogenized in 3 volumes of ice-cold 1.15% potassium chloride solution using a Teflon homogenizer. Homogenized samples were centrifuged at 9,000 × g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000 × g at 4°C for 70 min to obtain a mitochondria-free microsomal pellet. The washed microsomes were then suspended in 0.1 M potassium phosphate buffer (KPB), pH 7.4. The suspension was divided into 1.5-m*l* tubes, snap frozen in liquid nitrogen and kept at –80°C until use. Microsomal protein concentrations were determined by the spectrophotometric method described by Lowry *et al.* [16] using BSA as the standard. Total microsomal CYP was quantified from the carbon monoxide difference spectrum of the dithionite-reduced proteins by subtracting the values between 450 and 490 nm using an extinction coefficient of 91mM cm [21].

Assay of ethoxyresorufin and methoxyresorufin O-dealkylation (EROD and MROD) activities: The activities of ethoxyresorufin and methoxyresorufin O-dealkylation were determined by the method described by Burke *et al.* [2] with slight modifications. Briefly, one milliliter of the reaction mixture containing 10 mg microsomal protein, 10 mM G-6-P, 10 mM $MgCl_2$ and 20 μM ethoxyresorufin or methoxyresorufin in 0.1 M KPB (pH 7.4) was preincubated for 5 min at 37°C. The reaction was started by adding 20 μ*l* of a mixture of 50 mM NADPH and 200 U/m*l* of G-6-PDH. After incubation for 5 min, the reaction was terminated by adding 4 m*l* of cold methanol. The mixture was centrifuged at 3,000 rpm for 5 min, and the supernatant methanol layer was collected for measurement of resorufin. Resorufin was measured using a fluorescence spectrophotometer (FP777, Japan Spectroscopic Co., Tokyo, Japan). The excitation wavelength was set at 530 nm, and the emission wavelength was set at 590 nm.

Inhibition experiments of EROD and MROD: Antibody inhibition for both EROD and MROD was examined in this study using an anti-rat CYP1A1 antibody raised in rabbit that can recognize both isoforms of the CYP1A subfamily. The antibody was added to microsomes at a concentration of 2.5–10 μg/μ*l*, and the microsomes were then preincubated at room temperature for 30 min. In the control group, rabbit nonimmune serum was added instead of the antibody. The reaction was initiated by adding the reaction mixtures for the EROD and MROD assays to the microsomes preincubated with antibody or nonimmune serum, as mentioned previously [2].

Kinetic analysis of EROD: For the kinetic studies, EROD activity was determined over the substrate concentration range of 0.312–20.0 μM. Michaelis-Menten parameters, maximum velocity (Vmax) and Michaelis constant (Km) values were calculated from a hyperbolic regression curve fitted using a nonlinear least-squares regression by the GraphPad Prism 5 software (GraphPad Software Inc, La Jolla, CA, U.S.A.). Lineweaver-Burk plots were used to categorize the enzyme kinetics as mono or bi-phasic, i.e., whether one or more enzymes participate in the reaction, using the following formula.

$$1IV = [S] + Km/Vmax[S] = Km/Vmax \times 1/[S] + 1/Vmax$$

Statistical analysis: All data are expressed as means ± standard deviation (SD). Statistical significance was evaluated by Tukey-Kramer HSD difference test using JMP (SAS Institute, Cary, NC, USA). $P<0.05$ was considered to be significant.

RESULTS

EROD and MROD activities: Total CYP content was markedly higher in the rats compared with the contents of the cattle, deer and horses, respectively (Table 1). The EROD activities at the substrate concentration of 20 μM were 6-fold higher in the horse microsomes than in the rat microsomes. These activities were also significantly higher in the cattle and deer microsomes compared with the rat microsomes (Fig. 1A). In the case of the MROD assay at 20 μM of methoxyresorufin, the horse liver microsomes still had higher activity compared with those of the cattle and deer microsomes, but there was no significant difference compared with those of the rat microsomes (Fig. 1B).

Antibody-related inhibition of EROD and MROD activities: Anti-rat CYP1A1 antibody inhibited both EROD and MROD activities in all of the rats, deer, cattle and horses in a concentration-dependent manner. The inhibition was almost complete when 10 μg of antibody was used (Fig. 2).

Kinetic analysis of EROD activity: To determine the catalytic efficiency of CYP1A in the animal species examined in this study, we analyzed the kinetic parameters for EROD activity over a wide range of substrate concentrations ranging from 0.312 to 20.0 μM. The results showed that the

Table 1. Summary of the total cytochrome P450 content and the kinetic analysis of EROD activity in the deer, cattle and horses compared with the rats

Parameter	Rat	Deer	Cattle	Horse
Total P450 (nmol/mg protein)	0.89 ± 0.15^{a}	0.59 ± 0.15^{b}	0.77 ± 0.030^{a}	0.55 ± 0.020^{b}
Vmax (pmol/min/mg)	107.5 ± 10.54^{a}	241.2 ± 8.100^{b}	337.1 ± 2.400^{c}	918.2 ± 53.40^{d}
Km (μM)	2.84 ± 0.84^{a}	3.62 ± 0.65^{a}	2.97 ± 0.03^{a}	6.25 ± 0.51^{b}
Vmax/Km	0.038 ± 0.008^{a}	0.067 ± 0.009^{b}	0.11 ± 0.004^{c}	0.15 ± 0.003^{d}

The cytochrome P450 contents and kinetic parameters of ethoxyresorufin O-deethylation in microsomes from murine, cervine, bovine and equine livers were measured. The data represent the means ± SD for three animals from each species. Values with identical superscript letters are not significantly different from each other ($p<0.05$).

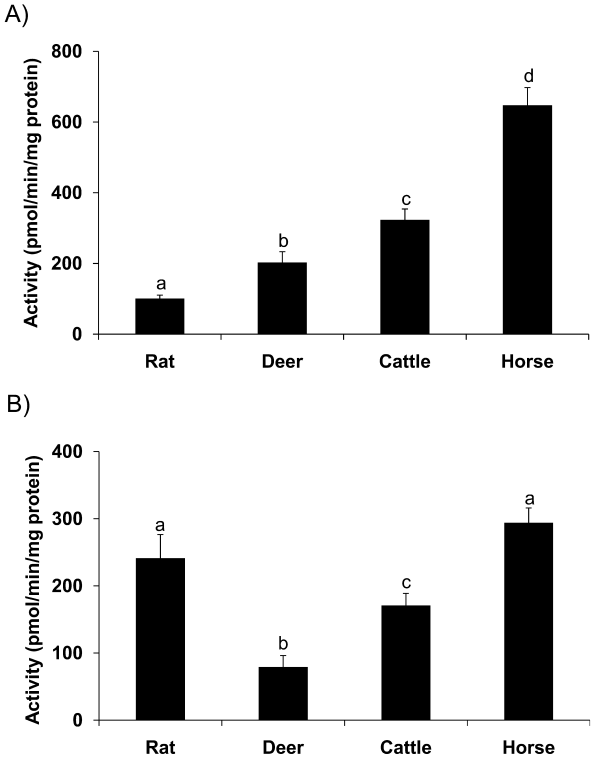

Fig. 1. CYP1A-dependent activities in liver microsomes of the deer, cattle and horses compared with those of the rats. EROD (A) and MROD (B) activities in the deer, cattle and horses compared with the rats using a substrate concentration of 20.0 μM for each. The data represent the means ± SD for three animals from each species. Values with identical superscript letters are not significantly different from each other ($p<0.05$).

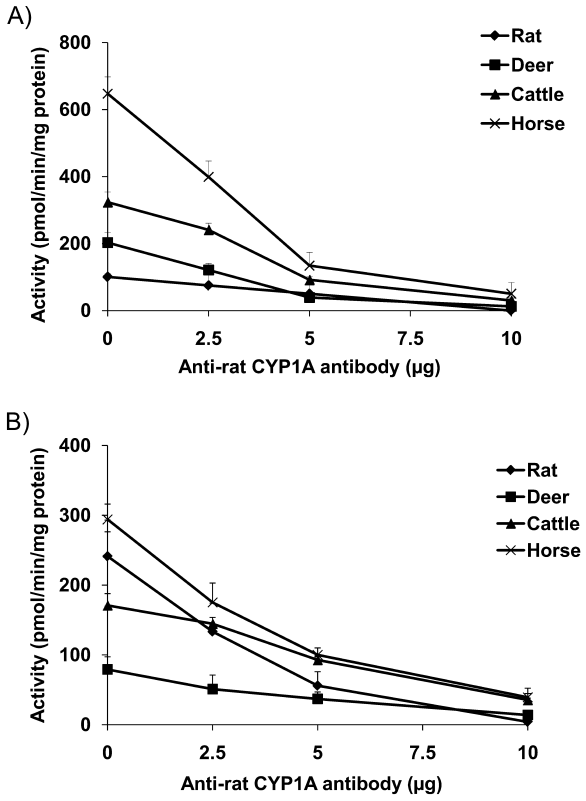

Fig. 2. Inhibition of EROD and MROD activities in liver microsomes of the deer, cattle and horses compared with those of the rats by anti-rat CYP1A1 antibody. Effects of anti-rat CYP1A1 antibody on EROD (A) or MROD (B) activity in the cattle, deer and horse hepatic microsomes compared with those of the rats at the same substrate concentration (20.0 μM) and different concentrations of the inhibitor, anti-rat CYP1A1 antibody (2.5, 5.0 and 10 μg) after incubation of both microsomes and the antibody for 30 min at room temperature. Data at the concentration of 0 represent the EROD activity without addition of an antibody. Rabbit IgG was used as a negative control instead of anti-rat CYP1A1 antibody in all animals; the data are not shown in the figure. Data represent means of three experiments performed at different times using three animals liver microsomes. Data at the concentrations of 2.5, 5.0 and 10 μg of the antibody are significant compared with the data at the concentration of 0.0 μg antibody in all animals ($p<0.05$).

equine hepatic microsomes had the highest Vmax (918.2 ± 53.4 pmol/min/mg) and highest catalytic efficiency (Vmax/Km; 0.15 ± 0.003 1/min/mg), although they also had the highest Km (6.25 ± 0.51 μM; Table 1). Cattle came second to the horses in terms of EROD catalytic efficiency (Vmax/Km value was 0.11 ± 0.004 1/min/mg) followed by deer (0.067 ± 0.009 1/min/mg). Although the rats showed the lowest Km value (2.84 ± 0.84 μM), which indicates high substrate affinity, they had the lowest EROD catalytic efficiency (Vmax/Km 0.038 ± 0.01 1/min/mg) due to the low Vmax value (107.5 ± 10.54 pmol/min/mg), as shown in

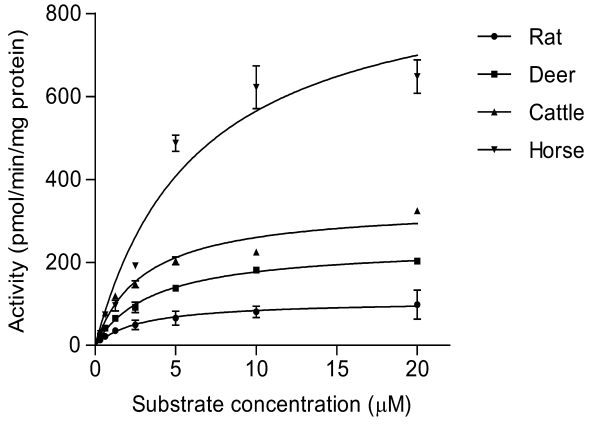

Fig. 3. Hyberbolic regression kinetic analysis for EROD activity in the deer, cattle and horses compared with the rats. Ethoxyresorufin O-deethylase activity was measured over substrate concentrations ranging from 0.312 to 20.0 μM. Data represent means of three experiments performed at different times using three animal liver microsomes.

Table1 and Fig. 3.

Analysis of the data utilizing Lineweaver-Burk plots revealed a slight curve in the EROD activity of the rats. However, we found a straight line for the Lineweaver-Burk plots within the substrate ranges studied in the ungulate animal species (Fig. 4).

DISCUSSION

The present study was undertaken to define CYP1A-dependent activities in meat producing animals like cattle, deer and horses compared to rats using EROD and MROD assays [9, 26]. In the equine liver microsomes, EROD showed higher activity compared with the rat, bovine and cervine microsomes, respectively. To confirm the high CYP1A-dependent activity in the horse liver microsomes, the catalytic activity of CYP1A towards methoxyresorufin, another ideal substrate for the CYP1A subfamily, was screened. The results for the MROD activity support our hypothesis that the horse has the highest CYP1A-dependent activities among the examined meat producing animals. These results suggest either greater expression of the CYP1A subfamily or that the horse had the highest catalytic preference towards both EROD and MROD activities compared with the other examined animal species. By comparing the EROD and MROD results in this study, it is clear that the rat had MROD activity that was greater than the EROD activity, probably due to a higher constitutive expression of CYP1A2, which is more active in MROD than CYP1A1 [3]. In contrast, the three meat producing animals examined had EROD activity that was higher than the MROD activity. This was more prominent in the wild population of deer, suggesting induction of CYP1A1 by environmental pollutants or by dietary inducers like flavonoids or carotenoids.

As far as we know, there is no information available on which CYP isoform is responsible for EROD and MROD

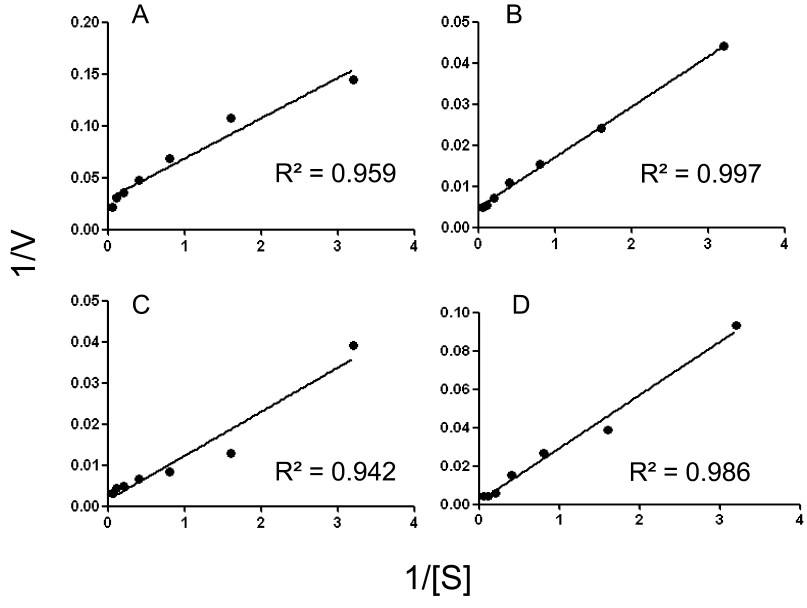

Fig. 4. Kinetic analysis of EROD activity using Lineweaver Burk plots in the deer, cattle and horses compared with the rats. Lineweaver Burk plots of ethoxyresorufin O-deethylase for the A) murine liver microsomes, B) cervine liver microsomes, C) bovine liver microsomes and D) equine liver microsomes. EROD activity was measured over substrate concentrations ranging from 0.312 to 20.0 μM. Data represent means of three experiments performed at different times using three animal liver microsomes.

activities in ungulates, including deer, cattle and horses. In previous reports [25, 26], α naphthoflavone was used as a chemical inhibitor for the CYP1A subfamily, but in fact, it is also a chemical inhibitor for other CYPs, like the CYP1B subfamily [11, 20]. Therefore, we tried to confirm our conclusion that the CYP1A subfamily is responsible for the aforementioned activities by monitoring an inhibition study for the EROD and MROD activities using anti-rat CYP1A1 antibody, which inhibits both CYP1A1 and CYP1A2 activities. We found that there is cross immunity between the rat and other examined animals, as the EROD activity was inhibited in a concentration-dependent phenomenon in all animals. The same phenomenon was repeated in the case of inhibition of MROD activity using the same antibody. These results highly suggest that EROD and MROD are specific activities for the CYP1A subfamily in the meat producing animals as well as in the rat. This is the first report accurately showing that EROD and MROD are CYP1A dependent activities in farm animals.

Due to the large interspecies difference in EROD activity, we studied the different kinetic parameters for EROD to clarify the characteristics of EROD reactions in these animals. Studying the kinetic parameters of EROD activity is a useful tool to investigate whether an interspecies difference is attributable to the difference in the concentration of CYP1A or due to an interspecies difference in enzyme efficiency.

Hyperbolic regression curves confirmed that the horse microsomes had the highest EROD activity over a wide range of substrate concentrations (0.312–20 μM), as the horse microsomes had the highest Vmax values compared with those of the cattle, deer and rats, respectively. The high Km values in the ungulate animals did not affect the high EROD activity in these animals compared with the rats, as the Vmax/Km values were still higher in these animals. In particular, the horse still had the highest enzyme efficiency as indicated by the highest Vmax/Km values. The enzyme efficiency of the horse microsomes was 4 times greater than that of the rat, bovine and cervine microsomes, respectively. We suggested that the horse liver possesses a high ability of CYP1A1-depedent metabolism at the clinical or physiological dose of substrates.

Further analysis for these parameters using Lineweaver-Burk plots revealed a straight line for the activity through the examined substrate concentrations in the ungulate species. In the case of the liver microsomes of the rats, Lineweaver-Burk plots made a slight curve and showed that another isoform, suggested to be CYP1A2, also contributed to part of the EROD reaction at a high concentration of substrate. We suggest that deethylation of ethoxyresorufin in ungulates, unlike that in rats, is due to a single enzyme or enzymes with similar Km values.

From these results, it can be concluded that the horse had the highest CYP1A-dependent activities in comparison to the deer, cattle and rats. This interspecies difference in EROD activity may be due to the difference of the CYP1A enzyme efficiency, but the interspecies difference in the CYP1A concentration cannot be ignored.

Cytochrome P450, especially the CYP1A subfamily content and activity, is genetically controlled and modulated by many physiological and environmental factors [8]. Age, pregnancy, external stimuli such as previous exposure to other chemicals [19] and the presence of disease affect CYP1A1-dependent activities [12]. Nutrition and diet composition have strong effects on the expressions of different P450s and xenobiotic metabolism [9, 22, 23]. Further approaches are needed to completely explain the interspecies difference in CYP1A-dependent activities.

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