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Carotenoids as regulators for inter-species difference in Cytochrome P450 1A expression and activity in ungulates and rats

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Abbreviations:

Cytochrome P450 (CYP), ethoxyresorufin O-deethylase (EROD), polycyclic aromatic hydrocarbons (PAHs), Naphthalene (Nap), Acenaphthene (Ace), Fluorene (Fle), Anthracene (Ant), Phenanthrene (Phe), Fluoranthene (Flu), Pyrene (Pyr), Benzo[*a*]anthracene (BaA), Chrysene (Chr), Benzo[*e*]pyrene (BeP), Benzo[*b*]fluoranthene (BbF), Benzo[*k*]fluoranthene (BkF), Benzo[*a*]pyrene (BaP), Dibenz[*a,h*]anthracene (DahA), Benzo[*ghi*]perylene (BghiP), potassium phosphate buffer (KPB), bovine serum albumin (BSA), sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), quantitative Real Time polymerase chain reaction (QRT-PCR), high performance liquid chromatography (HPLC), Aryl hydrocarbon Receptor (AhR).

Abstract

Ungulates (deer, cattle, and horses) are reported as animal species which show extreme-accelerated metabolism of CYP1A substrates, such as ethoxyresorufin compared to rats. This study was undertaken to investigate whether accumulation of carotenoids is a possible cause for inter-species difference in CYP1A-dependent activity in this group of animals. The relationship between inter-species differences in CYP1A-dependent activity and the accumulated carotenoids and retinoids as candidates of dietary CYP1A inducers in ungulate species was clarified. Interestingly, there were positive correlations between the accumulated carotenoids, such as β -carotene, with both EROD activity and CYP1A protein expression. These correlations were negative with the accumulated retinoids, such as retinol. The β -carotene was major component of carotenoids in ungulates, and known as an inducer of CYP1A. On the other hand, the retinol is reported as the reducer of CYP1A. Other factors which affect CYP1A1 expression, such as polycyclic aromatic hydrocarbons, were also analyzed. To cancel the effects of inter-species difference in CYP1A induction signal cascade among these animals, the rat cell line (H4-II-cells) was treated with the extracted carotenoids from the examined animals. In conclusion, carotenoids and retinoids may have direct effects on the inter-species differences in CYP1A-dependent activity and protein expression.

1. Introduction

The cytochrome P450 (CYP) superfamily is comprised of more than 5000 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 such as carotenoids and retinoids. Moreover, the CYP1A subfamily plays an important role in both mediating and mitigating the biological effects of these chemicals, which can determine susceptibility to toxicity or disease (Elskus et al., 1999; Gonzalez and Kimura, 2003; Teraoka et al., 2003; Heather et al., 2006).

Cytochrome P450, especially the CYP1A subfamily content and activity, is genetically controlled but can also be modulated by many other factors including age (Hulla and Juchau, 1989), pregnancy, external stimuli, such as previous exposure to other chemicals (Okey, 1990), and the presence of disease (Ioannides et al., 1996). Major factors affecting xenobiotic metabolism and P450 expression and activities are nutrition and diet composition (Parke and Ioannides, 1981; Parke, 1991; Ioannides, 1999). Studies performed on rats and humans have shown that both the CYP1A activity and its expression are affected by the components of diet such as protein components, lipids and lipotropes (Irizar and Ioannides, 1998), choline (Zhang et al., 1997), fibers (Kawata et al., 1992), and vitamins (Murray et al., 1991; Astorg et al., 1994). Synthetic flavonoids, such as β -naphthoflavone, are established potent and selective inducers of the CYP1A subfamily (Canivenc-Lavier et al., 1996a,b). Carotenoids, such as astaxanthin, canthaxanthin, β -carotene and bixin, have been found to induce CYP1A isoforms (Astorg et al., 1994; Jewell and O'Brien, 1999).

Catalytic selectivity of individual CYP enzymes have been compared for several rodent species, dogs, monkeys as well as humans (Weaver et al., 1994; Guengerich, 1997; Shimada et al., 1997; Anzenbacher et al., 1998 and Ioannides, 2006). Especially, previous research about the metabolic activity of CYP1A has shown that horses had a markedly higher CYP1A-dependent EROD activity compared to that of rats under low substrate concentration 0.8 μ M (Nebbia et al., 2003). Other reports have found that cattle and deer had higher CYP1A dependent activities compared to rats (Sivapathasundaram et al., 2001; Sivapathasundaram et al., 2003). However, until now, there have been no reports identifying the possible mechanisms behind the interspecies differences in the expression and activity of the CYP1A subfamily. Of particular note is the role of diet and accumulated dietary chemicals on that difference. At the same time, deer, cattle and horses are exposed during their lifetime to a host of xenobiotics such as drugs, growth promoters, and environmental contaminants.

The objective of this study is to investigate the role of the accumulated carotenoids and retinoids in the inter-species differences in CYP1A-dependent activity and protein expression among deer, cattle and horses and rats in a comparative way. The correlation between the accumulated carotenoids and retinoids, and the CYP1A dependent EROD activity and protein expression were investigated. To cancel the possibility of species difference of CYP1A induction signal cascade, a rat hepatic cell line was treated with the carotenoid extracts from the livers of the different examined animals.

2. Materials and Methods

2.1. Chemicals and reagents

All test reagents used were of reagent grade including those described below. Resorufin, ethoxyresorufin, bovine serum albumin (BSA), TRI reagent, and β -carotene were purchased from Sigma (St Louis, MO, USA). Retinol was obtained from Funakoshi Co. (Tokyo, Japan). NADPH, glucose-6-phosphate (G-6-P), and glucose-6-phosphate dehydrogenase (G-6-PDH) were from Oriental Yeast (Tokyo, Japan). Polyclonal rabbit anti-rat CYP1A1 antibody was from Daiichi Pure Chemical Co., Ltd. (Tokyo, Japan). Anti-rat CYP1A1 is reported to cross-react with CYP1A2 in rat microsomes. Standards of 15 PAHs (Naphthalene [Nap], Acenaphthene [Ace], Fluorene [Fle], Anthracene [Ant], Phenanthrene [Phe], Fluoranthene [Flu], Pyrene [Pyr], Benzo[*a*]anthracene [BaA], Chrysene [Chr], Benzo[*e*]pyrene [BeP], Benzo[*b*]fluoranthene [BbF], Benzo[*k*]fluoranthene [BkF], Benzo[*a*]pyrene [BaP], Dibenzo[*a,h*]anthracene [DahA], Benzo[*ghi*]perylene [BghiP]) were purchased from AccuStandard, Inc. (New Haven, USA). All other reagents were of analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries (Tokyo, Japan).

2.2. 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 five adult females of each species. Samples from Holstein cattle (*Bos taurus*) between the ages of four and five years (4.67 ± 0.58 year old) were purchased from Hokkaido University cattle farm. Samples from thoroughbred horses (*Equus caballus*) between the ages of four and six years (5.33 ± 1.15 year old) were kindly gifted from JRA (Japan Racing Association, Japan). Ezo shika deer (*Cervus hortulorum yesoensis*) were hunted from wild life (Hokkaido, Japan) during winter season, and their ages were estimated by tooth eruption and tooth wear patterns

(2.00±0.71 year old). Cattle and horses were reared under grass feeding with a non-medical history for at least one month prior to sacrifice. The livers of these ungulates were excised immediately after slaughter or hunting and were transferred to liquid nitrogen tanks. Nine week old Wistar female rats (SLC Hamamatsu, Japan) were housed at 24±1°C with 12 hr light and 12 hr dark cycles, and given a basal laboratory animal feed containing corn, soyabean, wheat and fish oil supplemented with vitamins and minerals (Labo MR stock, Nihon Nosan Kogyo Co.Ltd (Tokyo, Japan), and water ad libitum. Rats were anaesthetized and sacrificed with carbon dioxide. The rat livers, used as controls in this study, were removed and perfused with cold 1.15% KCl which removed the blood.

2.3. Cell line and culture condition

H4-II-E rat hepatoma cells obtained from the American Type Culture Collection (Manassas, VA), were grown in Dulbecco's Modified Eagle's Medium (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum and antibiotics (100 U/mL penicillin, 100 µg/mL streptomycin) at 37°C in a humidified incubator with 5 % CO₂ in air. Cells were seeded in 60 mm collagen- coated dishes, sub-cultured twice a week and subsequently grown to 80-90% confluence in 60 mm collagen-coated dishes. β-carotene, retinol (10⁻⁶ M) and different carotenoid extracts (1µg/µL) were added for 24h according to the method previously described by Kistler et al., (2002). Dimethyl sulphoxide (DMSO) was used as a negative control.

2.4. Preparation of liver microsomes

Liver microsomes from the examined deer, cattle, horses and rats, were prepared by the method described before by Omura and Sato, (1964) with slight modifications. 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 x g at 4°C for 20 min. The supernatant fraction was centrifuged at 105,000 x 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, and subsequently divided among 1.5 mL tubes, snap-frozen in liquid nitrogen, and kept at -80°C until use. Microsomal protein concentrations were determined using the method outlined by Lowery et al. (1951), using BSA as a standard.

2.5. Preparation of the whole cell lysate from H4-II-E rat cells

Whole cell lysate from H4-II-E rat cells were prepared according to the methods described by Birkner et al. (2003). The protein concentration in the whole cell lysates was measured using the method outlined by Lowery et al. (1951), using BSA as a standard.

2.6. Ethoxyresorufin O-deethylation (EROD) assay

EROD activity was determined by the method described before (Burke et al., 1985), with slight modifications. Briefly, one milliliter of the reaction mixture containing one milligram of protein, 10 mM G-6-P, 10 mM MgCl₂, and 2.5 μM ethoxyresorufin in 0.1 M KPB, pH 7.4, was preincubated for 5 min at 37°C. The reaction was started by adding 20 μL of a mixture of 50 mM NADPH and 200 U/mL of G-6-PDH. After incubation for 5 min, the reaction was terminated by adding 4 mL of cold methanol. The mixture was centrifuged at 3000 x g for 5 min, and the supernatant methanol layer was collected for measurement of resorufin. Resorufin was measured using a fluorescence spectrophotometer (FP777, Jasco). The excitation wavelength was set at 530 nm and the emission wavelength at 590 nm.

2.7. Western blot analysis

Polyacrylamide gel electrophoresis (SDS-PAGE) was conducted according to the method used by Laemmli, (1970). Using a 10% polyacrylamide gel, 15 μg of protein from each animal or treated cell culture was loaded in the lanes. After electrophoresis, the proteins in the gel were transferred to nitrocellulose membranes by an electrophoretic transfer method. The membrane was blocked with 5% skim milk, and the antigen-antibody reactions were carried out using goat

anti-rat CYP1A1 serum (diluted 1:500) as the first antibody, and a rabbit anti-goat IgG antibody conjugated with peroxidase (diluted 1:200) as the secondary antibody. Both antigen-antibody reactions were conducted for 1 hour at 37°C. After the antigen-antibody reaction, color was developed using a mixture of diaminobenzidine (0.025% 3,3-diaminobenzidine, 0.0075% Tris-HCl buffer, pH 7.4). Intensities of the immunoreactive bands were densitometrically analysed using the public domain NIH Image program (National Institutes of Health, MD, USA).

2.8. Extraction of total carotenoids and retinoids from livers of different animals

Carotenoids and retinoids were extracted from the livers of the examined animals according to the method used before (Yang et al., 1992). Briefly, one gram of liver was ground with 2-3 g sodium sulfate and 100 mg ascorbic acid to a uniform dry powder. This mixture was hydrolyzed in 3 mL of 20% KOH solution in ethanol, at room temperature, overnight, and in the dark. The carotenoid and retinoid extraction process was repeated three times with 3 mL n-hexane. The moisture in the extracts was removed using anhydrous sodium sulfite and an appropriate volume was evaporated under nitrogen. The residue was redissolved in ethanol and the absorption measured spectrophotometrically. A value of 2620 was used for the extinction coefficient ($E^{1\%}$) at 450 nm.

2.9. Extraction and HPLC analysis of β -carotene and retinol

The extraction of β -carotene and retinol from the liver of each animal was carried out by the method described previously (Hosotani and Kitagawa, 2003) with some modifications. This method is highly specific and yields higher concentrations of both β -carotene and retinol. Briefly, one gram of each liver was homogenated with 2.5 mL of 25% sodium ascorbate, 10 mL of ethanol, and 3.5 mL of water. After incubation at 70°C for 5 min, samples were saponified with 10 M KOH (5 mL) and heated at 70°C for 30 min. After cooling, n-hexane (10 mL) was added. The tubes were then vigorously shaken for 5 min and then centrifuged at 3000 x g for 5

min. This extraction process was repeated three times. The *n*-hexane extracts were dried by N₂ gas at 40°C, and the residue was dissolved in 1000 µL of methanol. The HPLC analysis was carried out using a Prominence LC-20 HPLC system (Shimadzu, Kyoto, Japan) with an UV detector (SPD-20A; Shimadzu, for β-carotene analysis) and a fluorescence detector (FD) (RF-10AXL; Shimadzu, for retinol analysis). The columns equipped with the HPLC, were the TSK-GEL ODS-120 (250 mm × 2.0 mm φ, (5 µm); Tosoh Co. Tokyo, Japan) for retinol and the L-column 2 ODS (150 mm × 2.1 mm φ, (5µm); CERI, Tokyo, Japan) for β-carotene. 5 µl aliquots of each sample were injected and separated using an isocratic solvent of 0.05% TFA water and 0.05% TFA methanol (15/85 (v/v) for retinol, and 2/98 (v/v) for β-carotene analysis) at a flow rate of 0.5 ml min⁻¹. The column temperature was kept at 35°C. For retinol analysis, excitation and emission wavelengths of the FD were set at 340 nm and 460 nm, respectively. For β-carotene analysis, the wavelength for UV was set at 451 nm.

2.10. RNA extraction

Total RNA was prepared from H4-II-E cells by the single-step method described by before (Chomczynski et al., 1987), using TRI reagent. The concentration and purity of the RNA fraction was determined spectrophotometrically at 260 and 280 nm, respectively.

2.11. Quantitative Real-Time RT-PCR

cDNA was synthesized as follows: a mixture containing 5 µg total RNA and 0.5 ng oligo dT primer was incubated in a total volume of 24 µL sterilized ultrapure water at 70°C for 10 min. This mixture was then removed from the thermal cycler and made up to 40 µL with 4 µL of (5x) RT-buffer, 8 µL of 10 mM dNTP, 2 µL of DEPC water and 2 µL of reverse transcriptase (TOYOBO Co. Ltd., Osaka, Japan). The mixture was then reincubated in the thermal cycler at 30°C for 10 min, 42°C for 1h and 90°C for 10 min to prepare the cDNA.

Quantitative Real-Time RT-PCR (Q RT-PCR) for rat CYP1A1, CYP2B1 and β -actin mRNA levels were performed using the Step One Plus Real-Time PCR system (Applied Biosystems, Foster, CA) and the DyNAmo HS SYBR Green qPCR kit (FINNZYMES Oy, Keilaranta, Finland). The instruction manual was followed with the use of the primer CYP1A1 sense CCATGACCAGGAACTATGGG and antisense TCTGGTGAGCATCCAGGACA (accession number X00469), CYP2B1 sense GCTCAAGTACCCCATGTCTG and antisense ATCAGTGTATGGCATTCTTACTGCGG (accession number NP-001128316) and the β -actin sense ATGTACGTAGCCATCCAGGC and antisense TCCACACAGAGTACTTGCGC (accession number V01217). The reaction mixture (final volume 20 μ L) for the PCR was prepared with 1x Master Mix reagents, 300 nM of each primer, 500 ng cDNA, 1x ROX reference dye in 1 μ L RNase-free water for rat CYP and β -actin. The mixture was completed to the final volume by RNase-free water. The reaction was performed for 40 cycles: initial activation at 95°C for 15 min, denaturation at 95°C, annealing at 60°C for 1 min for CYP1A1 and at 62°C for 1 min for CYP2B1, and at 72°C for 30 sec. The measurement of each treatment was performed in duplicate and repeated three times. The expression of CYP1A1 was normalized to the expression of β -actin and was calculated relative to that of rat carotenoid extract treatment.

2.12. Analysis of PAHs

PAHs were analyzed by methods described before (Ikenaka et al., 2008) with slight modifications. Briefly, about 10 g of each liver sample was homogenized with approximately 20 mL of alkaline solution (1 M KOH ethanol solution), and saponified for 12 h at 55°C. The saponified solutions were then shake-extracted 3 times with n-hexane and the resulting hexane fractions containing the PAHs were run through granular sodium sulphate. After evaporation by using a rotary evaporator, the hexane fractions were purified using silica gel column chromatography (2 g of 5% water containing silica gel). The PAHs fractions were eluted by 100

mL of Acetone/ Hexane = 1/99 (v/v). The PAHs fractions were dried under a gentle nitrogen stream, and re-dissolved into 0.5 mL of methanol for HPLC analysis. HPLC analyses were performed using a Shimadzu LC20 series (Kyoto, Japan) equipped with a fluorescence detector (RF-10AxL) and a ZORBAX Eclipse PAH (2.1×150 mm, 3.5µm, Agilent). Quantification of 15 PAHs (Naphthalene [Nap], Acenaphthene [Ace], Fluorene [Fle], Anthracene [Ant], Phenanthrene [Phe], Fluoranthene [Flu], Pyrene [Pyr], Benzo[*a*]anthracene [BaA], Chrysene [Chr], Benzo[*e*]pyrene [BeP], Benzo[*b*]fluoranthene [BbF], Benzo[*k*]fluoranthene [BkF], Benzo[*a*]pyrene [BaP], Dibenz[*a,h*]anthracene [DahA], Benzo[*ghi*]perylene [BghiP]) were determined from calibration curves made by the standards (Accus Standard Inc.).

2.13. Statistical analysis

All data are expressed as mean ± standard deviation (SD). Statistical significances were evaluated by Tukey's Kramer HSD difference test using JMP (SAS Institute, Cary, NC, USA). $P < 0.05$ was considered to be significant.

3. Results

3.1. Results of the EROD activity in the examined ungulates and rats

The O-deethylation of ethoxyresorufin was strongly higher in horse microsomes, about six times than in rats, and also significantly higher in cattle and deer microsomes compared to that of rats (Fig 1A).

3.2. Protein expression in the examined ungulates and rats

Ungulate microsomes showed immune-reactivity to anti-rat CYP1A1 in the Western blot analysis. Horse microsomes had the highest band intensities followed by cattle, deer, and rats, respectively (Fig. 1B).

3.3. Carotenoid contents in livers of the examined ungulates and rats Total carotenoid content was measured spectrophotometrically in the livers of the examined animals. Horses and cattle had significantly higher content than deer and rats. The carotenoid content of liver tissue in horses was 6.8 ± 0.6 , cattle 7.6 ± 0.5 , deer 3.7 ± 0.2 and rats 0.9 ± 0.2 $\mu\text{g/g}$ (Fig. 2A). Scatter plots between carotenoid content and EROD activity as well as between carotenoid content and protein expression levels in the livers of these animals were linear and had a positive correlation between each pair (Fig. 2B, C).

3.4. β -carotene content in livers of the examined ungulates and rats

HPLC analysis of β -carotene in the livers of the examined animals revealed that cattle (6.9 ± 0.7 $\mu\text{g/g}$) and horses (6.4 ± 0.6 $\mu\text{g/g}$) had the highest content of β -carotene followed by deer (3.6 ± 0.2 $\mu\text{g/g}$). No β -carotene was detected in the livers of the examined rats (Fig. 3A). Scatter plots between β -carotene content and EROD activity as well as the protein expression level of CYP1A1 showed positive correlations (Fig. 3 B, C).

3.5. Retinol content in livers of the examined ungulates and rats

HPLC analysis of retinol in the livers of the examined animals showed that rats had the highest content of retinol ($234.3 \pm 19 \mu\text{g/g}$) followed by cattle ($151.2 \pm 15.9 \mu\text{g/g}$), horses ($61.6 \pm 6.3 \mu\text{g/g}$) and deer ($58.3 \pm 13.7 \mu\text{g/g}$) (Fig. 4A). Scatter plots between the retinol content and both EROD activity and protein expression levels of CYP1A1 showed negative correlations (Fig. 4 B, C).

3.6. CYP1A1 and CYP2B1 mRNA expression in the H4-II-E cells

The CYP1A1 mRNA expression in the H4-II-E rat cells treated with the carotenoid extracts of the horse livers showed significantly higher expression than that extracted from other ungulates and rat. The induction level was nearly similar to that produced by β -carotene. Rat cells treated with carotenoid extract from bovine and cervine livers showed higher CYP1A1 mRNA expression compared to those produced by rat carotenoid extract. Retinol treated rat cells showed lower expression compared to non- treated cells (Fig. 5A). A positive correlation was seen between the scattered plots of the CYP1A1 mRNA expression in the treated cells and total carotenoid content in these extracts (Fig. 5B). In contrast, neither rat cells treated with different carotenoid extracts, nor those treated with β -carotene and retinol showed any significant difference of CYP2B1 expression with the untreated ones (Data are not shown).

3.7. CYP1A1 protein expression and EROD activity in the H4-II-E cells

The CYP1A1 protein expression in the H4-II-E rat cells treated with the different carotenoid extracts goes in line with the results of CYP1A1 mRNA expression. As the carotenoid extracts of the horse livers showed significantly higher expression than that extracted from rat. The induction level was nearly similar to that produced by β -carotene. Rat cells treated with carotenoid extract from bovine and cervine livers showed higher CYP1A1 protein expression compared to the non-treated cells. Retinol treated rat cells did not show any effect compared to non- treated cells (Fig. 6A). The results of the EROD activity in the H4-II-E rat cells treated

with carotenoid extracts of the horse livers showed significantly higher activity than all other treatments (Fig. 6B).

3.8. PAHs accumulation in the livers of the examined ungulates and rats

The liver of each of the different ungulates, as well as the liver of the rat, showed very low accumulated levels of the 15 measured PAHs. (Table 1).

4. Discussion

Our study defines the metabolic activity and protein expression of CYP1A in meat-producing animals such as cattle, deer and horses, with the rat as the control. EROD activity is routinely used to monitor CYP1A activity (Ioannides, 1999; Sivapathasundaram et al., 2003). In the equine liver, ethoxyresorufin O-deethylase showed the highest activity when compared to the rat, followed by the bovine and cervine microsomes, suggesting that the equine liver had the greatest expression of the CYP1A subfamily.

Using an immunochemical approach, it was determined that ungulates had a higher CYP1A protein expression compared to that of the rat. In particular, the horse microsomes had the highest intensity bands. In line with our results, proteins cross-reacting with antibodies raised against rat or rabbit CYP1A have been found in liver microsomes from cattle (Sivapathasundaram et al. 2001), pig (Myers et al. 2001), and horse (Nebbia et al. 2003), and the degree of their expression has been found to be related to the rate of the *in vitro* O-dealkylation of ethoxyresorufin.

In a parallel study, EROD kinetic parameters were investigated (Darwish et al., 2010), but we did not find marked differences in the catalytic efficiency between the animals examined. As the V_{max}/K_m values were 0.15 ± 0.003 , 0.11 ± 0.004 , 0.067 ± 0.009 , 0.038 ± 0.01 1/min/mg in horse, cattle, deer and rat liver microsomes respectively. These results suggest that the high CYP1A protein expression and dependent activities in ungulates is mainly due to the induction of CYP1A by dietary chemicals or environmental pollutants. Of particular note is the fact that all of these animals are herbivores and depend mainly on grass feeding. Hence there is a good chance for contamination of their food source by inducers of CYP1A like polycyclic aromatic

hydrocarbons. Alternatively, this food source may contain natural substances, such as carotenoids, that may also induce CYP1A activity (Ioannides 2002).

By analysis of 15 PAHs in the livers of the examined animals, as shown in Table 1, very low contaminant levels were observed compared to the recommended levels by the European Union no. 208/2005. These results strongly suggest the minimum contribution of these chemicals in the inter-species differences in the CYP1A-dependent activity and expression. Machala et al., (2003) also found that deer had a higher CYP1A-dependent activity compared to the rat. By analysis of the samples for different pollutants that may induce CYP1A, they found negative results for the chemical pollution.

The other source of induction of CYP1A is through dietary substances such as flavonoids like β -naphthoflavone (Parke, 1991; Canivenc-Lavier et al., 1996a, 1996b). In addition, some carotenoids such as astaxanthin, canthaxanthin, β -carotene and bixin are considered as inducers for CYP1A (Jewell and O'Brien, 1999). Especially, we focused on the carotenoids, because generally some of ungulates were reported as typical carotenoid-accumulators unlike the rats (Mathews-Roth et al., 1990; Slifka et al., 1999). Thus, we investigated the inter-species difference in the accumulation of carotenoids, the naturally occurring chemicals in many food stuffs available for these animals. The carotenoid content in the liver of these animals confirmed our new idea, as we found that horses and cattle had a higher content of the total carotenoids compared to the deer and rats. Horses and certain breeds of cattle have the ability to absorb some carotenoids as β -carotene intact as well as convert it to vitamin A (Bondi and Sklan, 1984; Mathews-Roth et al., 1990; Slifka et al., 1999), however, there is no information about the carotenoid accumulation in deer. In our study, the carotenoid content in the liver of these animals showed a markedly positive correlation with the protein expression of CYP1A as well as the CYP1A-dependent EROD activity.

As evidence for this new aspect, the accumulation levels of some individual carotenoids and retinoids such as β -carotene, retinol, astaxanthin, canthaxanthin, retinoic acid, and β -apo-8 carotenal were measured using HPLC.

Astaxanthin, canthaxanthin, retinoic acid, and β -apo-8 carotenal were not detected, which may be due to the very low levels in the livers of the examined animals. However, β -carotene and retinol were detected in these ungulates. Interestingly, β -carotene did not accumulate in rats, while cattle and horses showed the highest accumulation levels. Scatter plots of the β -carotene content, CYP1A protein expression, and EROD activity in these animals showed strong positive correlations, suggesting that the accumulation of β -carotene in these animals may lead to induction of CYP1A in these ungulates. This is of particular importance in light of the fact that β -carotene is reported to be an inducer for CYP1A1 both *in-vitro* and *in-vivo* as mentioned by Paolini et al., (1999, 2001). The mechanism of the induction of CYP1A1 expression and dependent activities by carotenoids in ungulates is still unclear, but it was reported that some carotenoids such as canthaxanthin or β -apo-8"-carotenal induced Cyp1A genes in mice through an Ah receptor-dependent pathway, but did not bind to the Ah receptor (Gradelet et al., 1997).

In contrast, the accumulation level of retinol was the highest in rats compared to the ungulates. This may indicate that all of the β -carotene was converted in to retinol in the case of rats. Especially, that rat diet contained wheat, corn, soyabean and fish oil which are rich in β -carotene content in contrast to ungulates which received only a grass feeding. Interestingly, a negative correlation between the accumulation of retinol with both CYP1A protein expression and EROD activity was found. This result may suggest that CYP1A induction, by carotenoids, reduces the levels of retinol in ungulates. This explanation corresponds with Besselink et al. (1998), who reported that exposure to CYP1A inducers (PAHs) decreases retinol levels in fish liver. Also, this result may suggest that the high accumulation of retinol in rats may lead to either reduction or inhibition of CYP1A expression and activity in rats

compared to ungulates, especially since retinol has been shown to inhibit CYP1A1 in 3-methylcholanthrene treated rats, as reported by Huang et al., (1999). Likely, it was reported that retinol and retinoic acid were strong inhibitors for xenobiotic oxidations catalysed by recombinant CYP1A1 and CYP1A2 through a competitive inhibition (Yamazaki and Shimada, 1999). The mechanism of reduction or inhibition of CYP1A expression and dependent metabolism by retinol and retinoic acid in ungulates is still unclear but the published reports, using human or rodent hepatic tissues, about this phenomenon strengthens our hypothesis. Chen et al. (2000) reported that CYP1A1 and CYP1A2 were highly active for catalyzing conversion of all-*trans*-retinol to all-*trans*-retinal and after that to all- *trans*-retinoic acid in the human hepatic tissue. Similar results were obtained in guinea pigs, hamsters, mice and rats (Fletcher et al., 2001). To confirm this new hypothesis, rat cells were exposed to different carotenoid extracts, in addition to β -carotene and retinol, which were used as control chemicals. We used rat hepatocytes in this assay, since we wanted to measure the effects of extracted carotenoid fractions from each animal, without the effects of the species differences in AhR function or in any other signal cascade which affect CYP1A expression. Surprisingly, the carotenoid extract from equine livers could induce CYP1A1 mRNA expression in the rat cells, followed by that extracted from bovine livers. The induction level was found to be nearly similar to that produced by β -carotene. Although bovine livers had a similar accumulation pattern of β -carotene to that of the horse, they were shown to accumulate retinol, which may interfere with the induction of CYP1A produced by β -carotene. Unlikely, CYP2B1 mRNA expression, which is not regulated by Ah receptor, did not show any significant difference in all treatments. Similar results were obtained in vivo by Nebbia et al. (2003) who did not find any significant difference in the CYP2B1 protein expression and dependent metabolism in cattle and horse liver microsomes. This result may suggest that the induction of CYP1A1 mRNA in the rat cells treated with different carotenoid extracts may occur through Ah receptor dependent passway.

In line with the induction of CYP1A1 mRNA in the rat cells treated with different carotenoid extracts, the CYP1A1 protein expression and dependent EROD activity in the H4-II-E rat cells treated with different carotenoid extracts, especially in horses, showed the same behavior. These results confirm the induction of CYP1A1 by different carotenoid extracts at both the pretranscriptional and post translational levels.

In conclusion, we suggest the accumulated carotenoids and retinoids such as β -carotene and retinol in the hepatic tissues of the ungulates and rats have a very important role at least in part, in the regulation of the inter-species difference in CYP1A dependent activity and protein expression. That means, the inter-species difference in the accumulation pattern of carotenoids and retinoids may explain the difference in CYP1A expression and activity among the examined ungulates and rats. Further approaches are needed to investigate the transcriptional mechanisms behind either induction or reduction of CYP1A by the accumulated carotenoids and retinoids. **Acknowledgments**

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Figure legends

Figure 1. CYP1A dependent EROD activity and protein expression in ungulates and rats.

A) EROD activity (pmol/min/mg protein) in different ungulates compared to rats using a substrate concentration of 2.5 μ M. The data represent the mean \pm SD for five animals from each species. Identical letters were not significantly different from each other. $P < 0.05$. B) CYP1A protein expression in different ungulates compared to rats. Microsomal protein samples (15 μ g/lane) were applied to 10% SDS-PAGE wells, transplotted onto nitrocellulose membranes, and reacted with a goat-anti rat CYP1A1 polyclonal antibody. Protein expression was analyzed relative to the rat. Intensities of the immunoreactive bands were densitometrically analyzed using the public domain NIH Image program.

Figure 2. Relationship between total carotenoid content and CYP1A.

A) Total carotenoid content in the liver of several ungulates as well as in rats. The total carotenoid content (μ g/g) was measured spectrophotometrically. A value of 2620 was used as the extinction coefficient ($E^{1\%}$) at 450 nm. The data represent the mean \pm SD for five animals from each species. Identical letters were not significantly different from each other. $P < 0.05$. B) Scatter plots between the total carotenoid content and EROD activity (pmol/min/mg protein) in the examined ungulates and in rats. C) Scatter plots between the total carotenoid content (μ g/g liver tissue) and CYP1A protein expression level in the ungulates and in rats.

Figure 3. Relationship between β -carotene content and CYP1A.

A) β -carotene content ($\mu\text{g/g}$) was measured by HPLC using a Prominence LC-20 HPLC system with an UV detector. The column equipped with HPLC was L-column 2 ODS ($150\text{ mm} \times 2.1\text{ mm } \phi$, $5\mu\text{m}$). $5\ \mu\text{l}$ aliquots of each sample were injected and separated using an isocratic solvent of 0.05% TFA water and 0.05% TFA methanol 2/98 (v/v) at a flow rate of 0.5 ml min^{-1} . The column temperature was kept at 35°C . The UV wavelength was set at 451 nm. The data represent the mean \pm SD for five animals from each species. Identical letters were not significantly different from each other. $P < 0.05$. B) Scatter plots between β -carotene content ($\mu\text{g/g}$ liver tissue) and EROD activity ($\text{pmol/min/mg protein}$) in the examined ungulates and in rats. C) Scatter plots between β -carotene content ($\mu\text{g/g}$ liver tissue) and CYP1A protein expression levels in the examined ungulates and in rats.

Figure 4. Relationship between retinol content and CYP1A.

A) Retinol content ($\mu\text{g/g}$) was measured by HPLC using a Prominence LC-20 HPLC system with a fluorescence detector (FD). The column equipped with HPLC was a TSK-GEL ODS-120 ($250\text{ mm} \times 2.0\text{ mm } \phi$, $5\ \mu\text{m}$). $5\ \mu\text{l}$ aliquots of each sample were injected and separated using an isocratic solvent of 0.05% TFA water and 0.05% TFA methanol (15/85 (v/v) at a flow rate of 0.5 ml min^{-1} . The column temperature was kept at 35°C . Excitation and emission wavelengths for FD were set at 340 nm and 460 nm, respectively. The data represent the mean \pm SD for five animals from each species. Identical letters were not significantly different from each other. $P < 0.05$. B) Scatter plots between retinol content ($\mu\text{g/g}$ liver tissue) and EROD activity ($\text{pmol/min/mg protein}$) in the examined ungulates and in rats. C) Scatter plots between retinol content ($\mu\text{g/g}$ liver tissue) and CYP1A protein expression levels in the examined ungulates and in rats.

Figure 5. CYP1A1 mRNA expression in H4-II-E rat cells and its correlation with total carotenoid content in the liver of various the examined ungulates as well as rats.

A) Effect of different carotenoid extracts, β -carotene, and retinol on CYP1A1 mRNA expression in H4-II-E rat cells using real-time RT-PCR analysis of CYP1A1 mRNA. The cDNA samples were amplified as described in the Materials and Methods section. The amount of CYP1A1 mRNA was normalized to the corresponding amount of β -actin and presented relative to the cells treated with rat carotenoid extract. Each treatment is represented by five plates. Data are presented as mean \pm SD. Identical letters were not significantly different from each other. $P < 0.05$. B) Scatter plots between total carotenoid content ($\mu\text{g/g}$ liver tissue) and CYP1A1 mRNA expression in the treated H4-II-E rat cells with different carotenoid extracts.

Figure 6. CYP1A protein expression and dependent EROD activity in H4-II-E rat cells.

A) CYP1A protein expression in H4-II-E rat cells treated with different carotenoid extracts. Whole cell lysate protein samples ($15\mu\text{g/lane}$) were applied to 10% SDS-PAGE wells, transplotted onto nitrocellulose membranes, and reacted with a goat-anti rat CYP1A1 polyclonal antibody. Protein expression was analyzed relative to the non-treated rat cells. Intensities of the immunoreactive bands were densitometrically analyzed using the public domain NIH Image program. B) EROD activity ($\text{pmol/min/mg protein}$) in H4-II-E rat cells treated with different carotenoid extracts.using a substrate concentration of $2.5\ \mu\text{M}$. The data represent the mean \pm SD for five treatments from each extract. Identical letters were not significantly different from each other. $P < 0.05$.

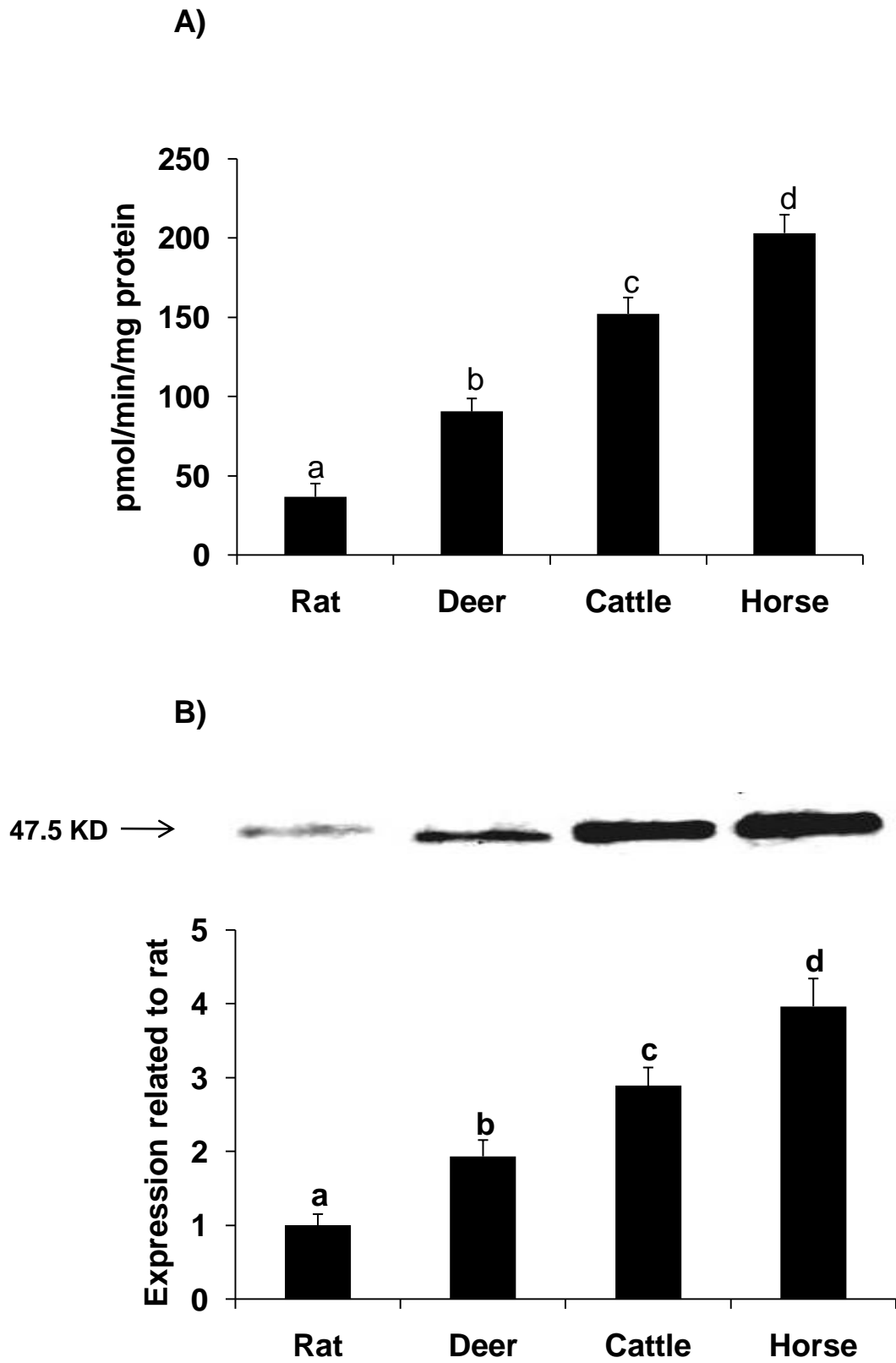


Figure 1

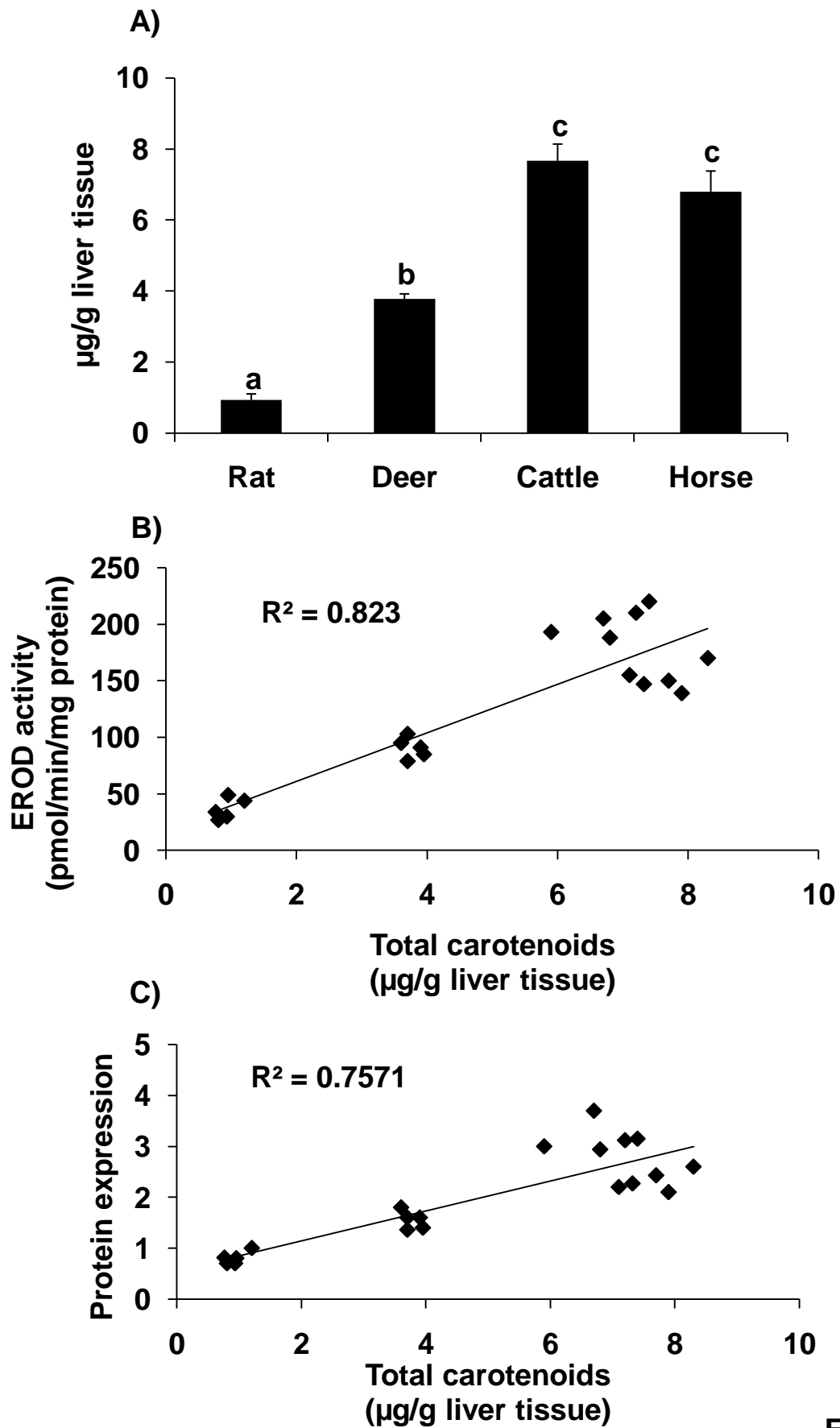


Figure 2

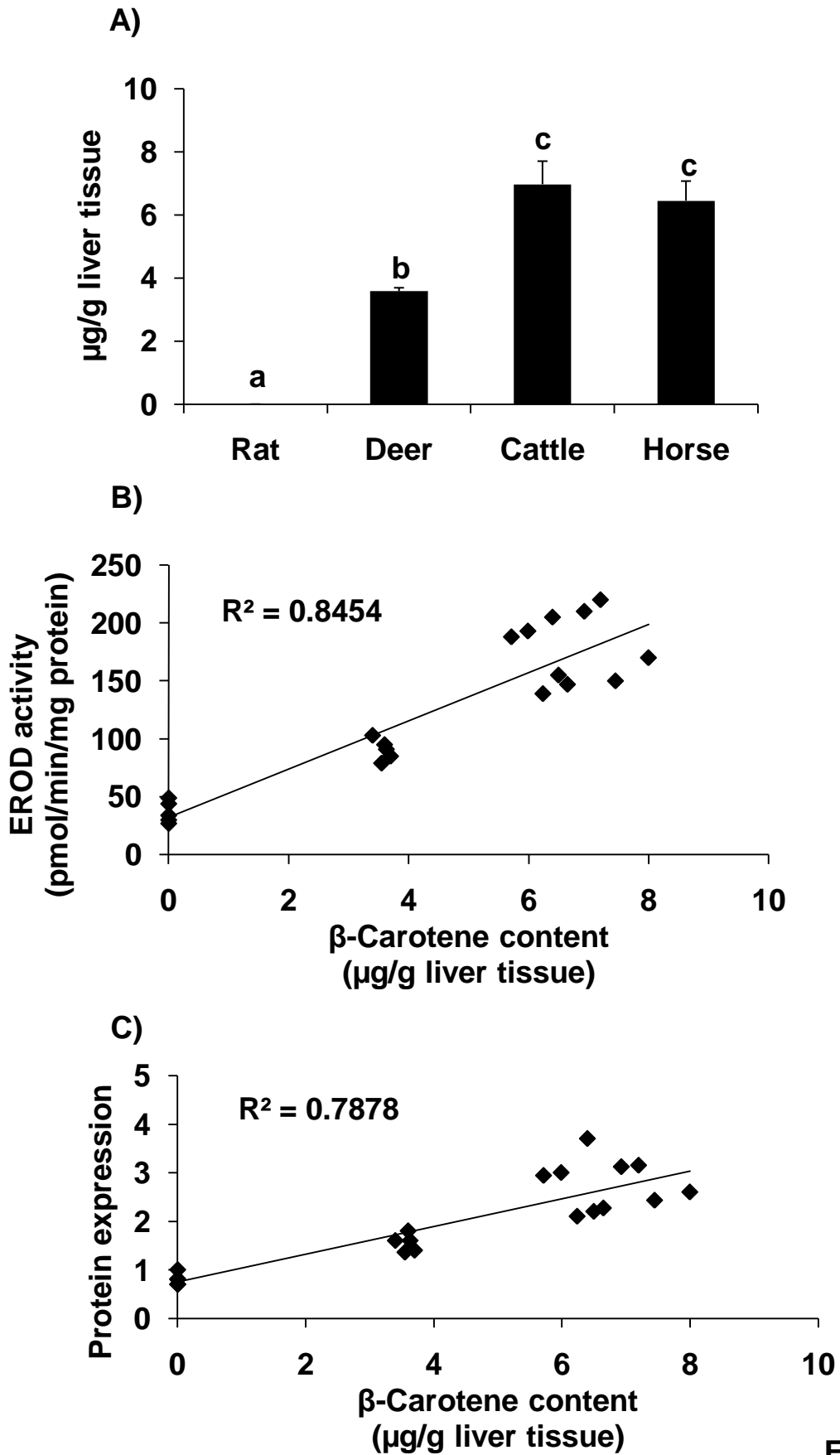
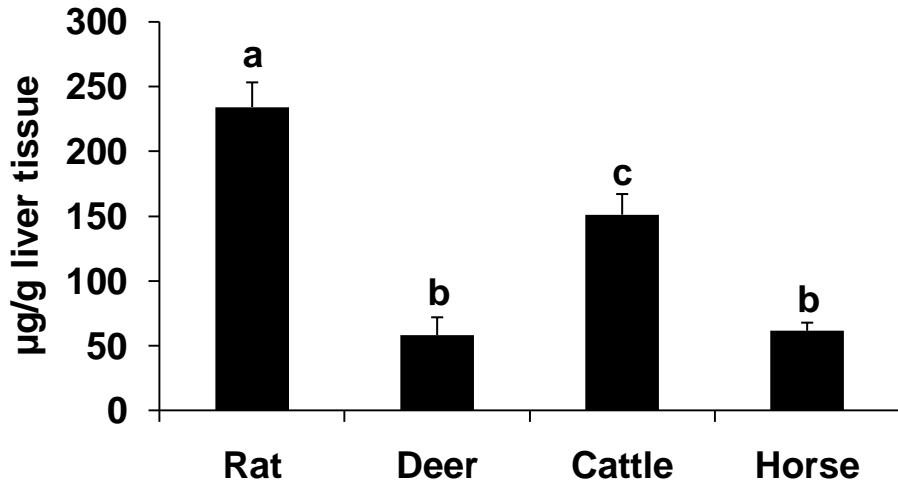
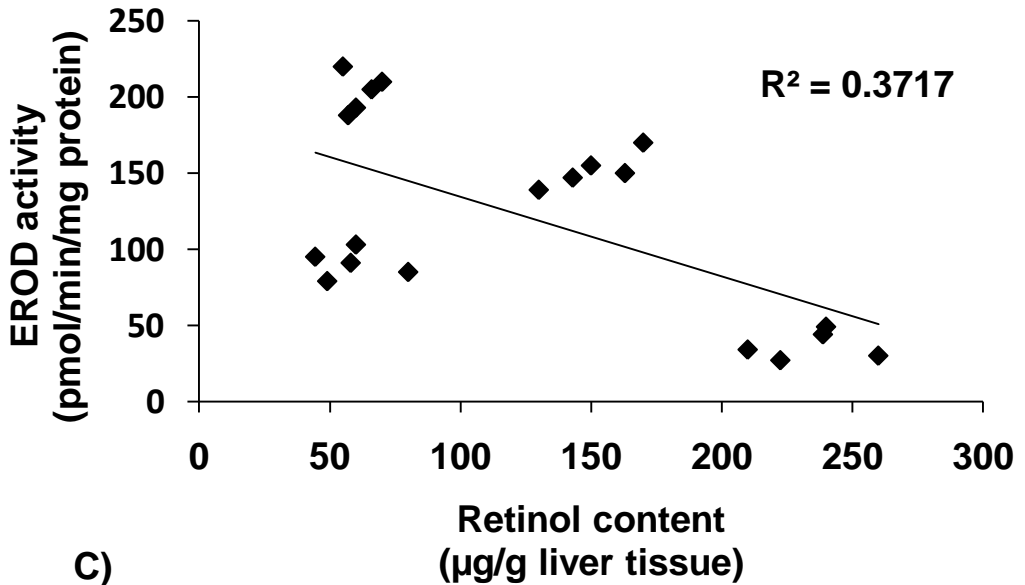


Figure 3

A)



B)



C)

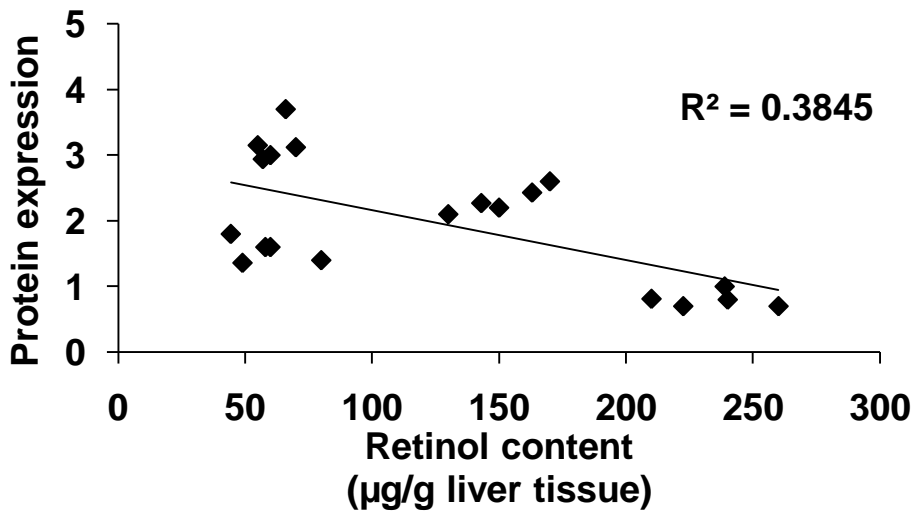


Figure4

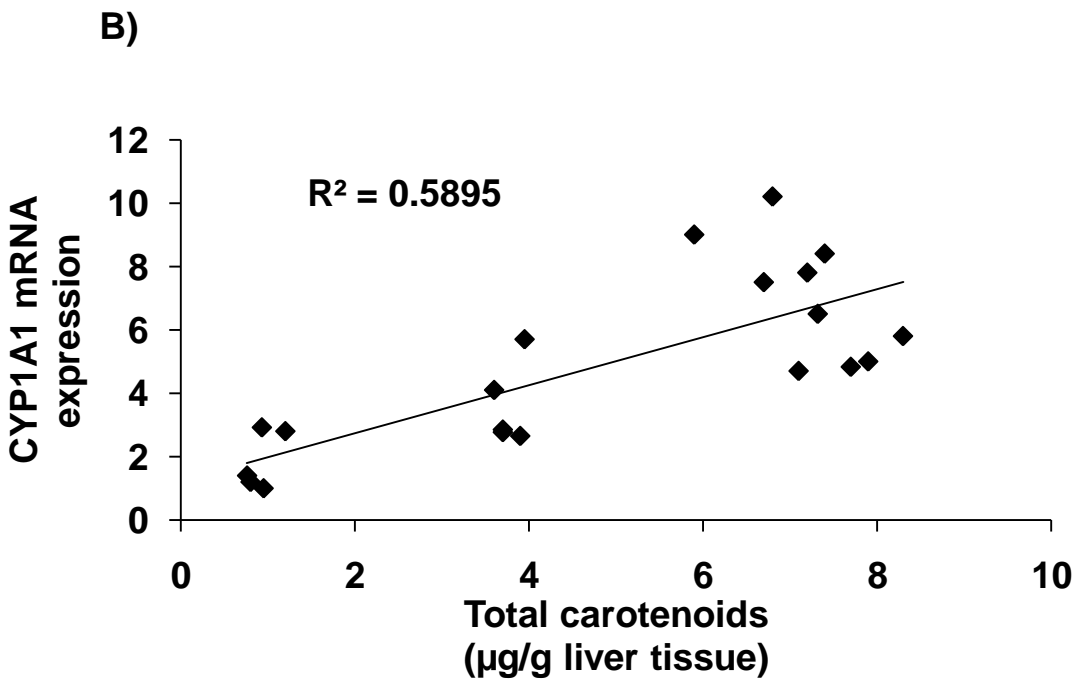
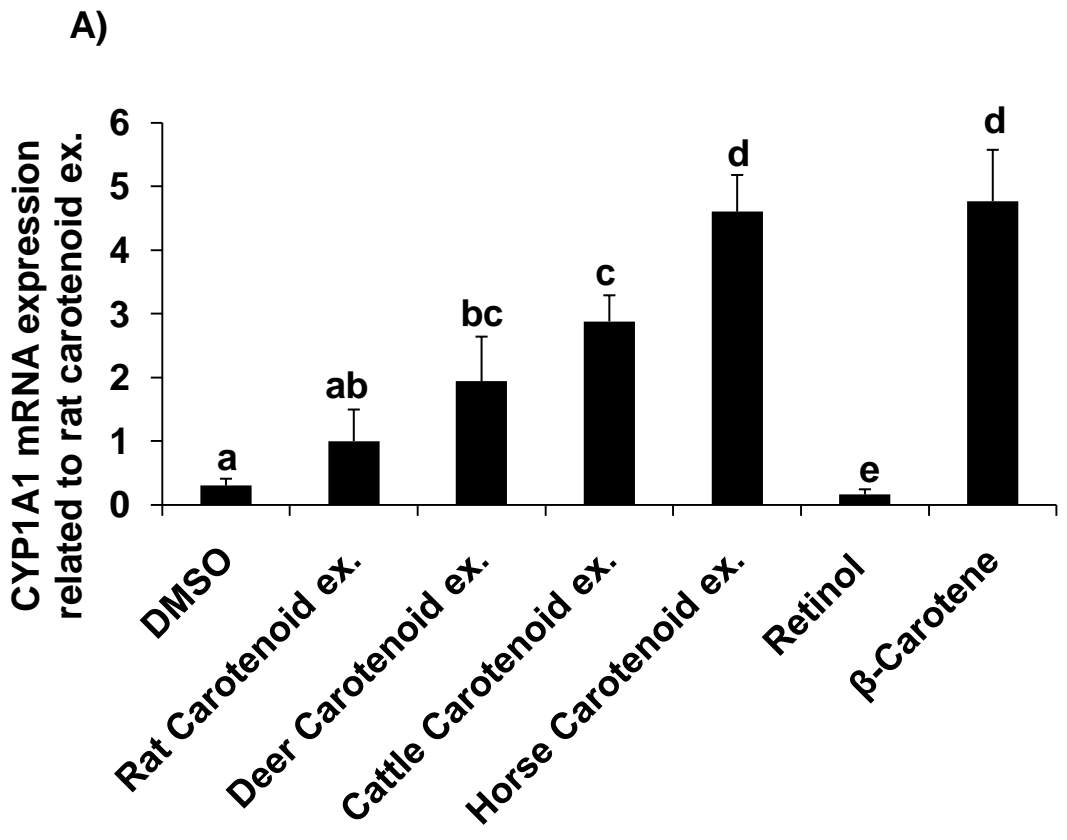


Figure 5

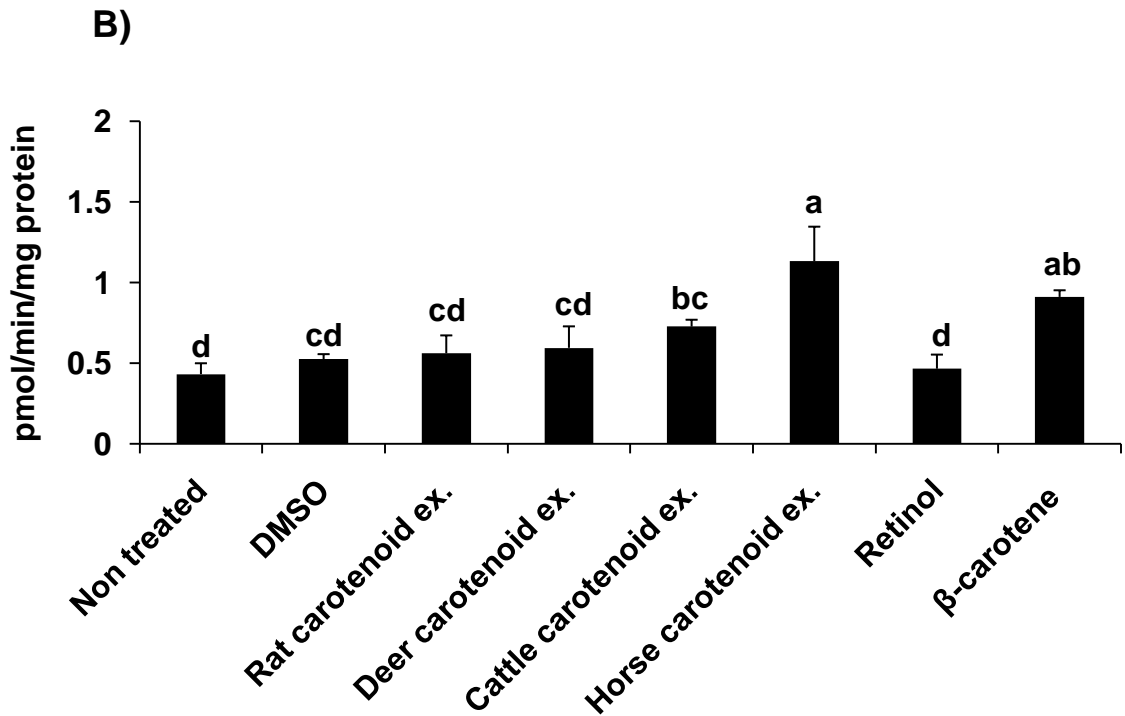
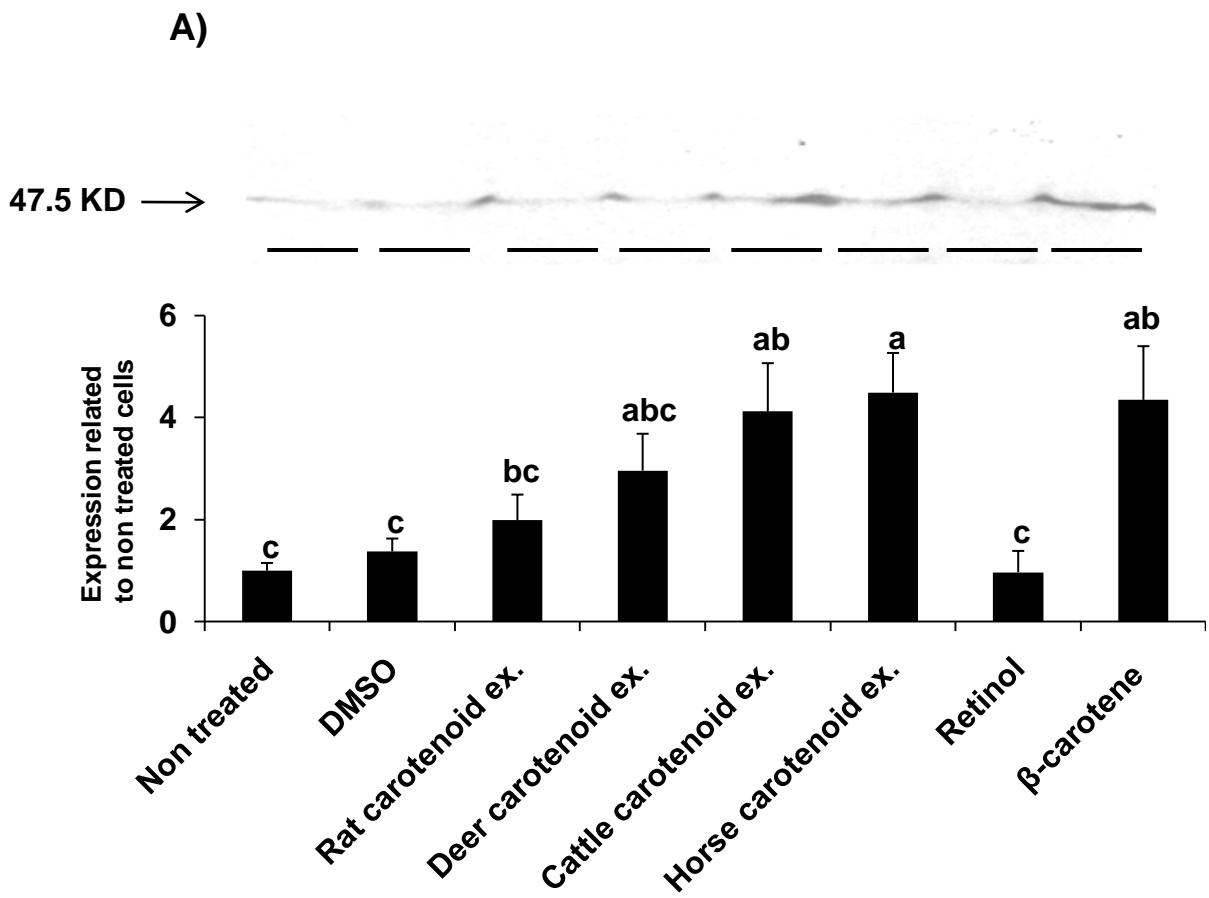


Figure 6

Table 1: Accumulated PAHs (ng/g) in the livers of examined ungulates as well as rats.

	Rat	Deer	Cattle	Horse
Ace	0.04±0.22	0.29±0.14	0.37±0.32	0.18±0.13
Fle	4.91±2.95	0.16±0.05	0.26±0.22	0.67±0.53
Anth	0.42±0.04	0.46±0.18	0.50±0.38	1.24±0.76
Phe	1.06±0.23	2.66±1.18	1.75±1.04	3.64±1.53
Flu	0.02±0.00	0.17±0.04	0.19±0.20	0.21±0.15
Pyr	0.49±0.06	0.90±0.21	1.77±0.59	3.00±1.53
BaA	0.14±0.02	0.40±0.09	0.54±0.13	0.72±0.24
Chry	0.49±0.01	0.20±0.14	0.09±0.11	0.05±0.03
BeP	0.14±0.01	0.23±0.24	0.09±0.03	0.06±0.03
BbF	0.18±0.01	0.24±0.12	0.06±0.08	0.02±0.00
BkF	0.55±0.02	0.55±0.15	0.13±0.00	0.33±0.13
BaP	0.01±0.004	0.09±0.01	0.01±0.00	0.02±0.01
DahA	0.38±0.11	0.09±0.08	0.01±0.00	0.01±0.01
BghiP	0.71±0.25	0.06±0.09	0.00±0.00	0.01±0.01
IDP	0.31±0.25	0.05±0.08	0.00±0.00	0.00±0.00

The data represent the means ± SD for five animals from each species.