Biological defense system against xenobiotics in meat-producing animals

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Contents

ABBREVIATIONS

Ace: Acenaphthene

AhR: Aryl hydrocarbon receptor

Ant: Anthracene

B[*a*]A: Benzo[*a*]anthracene

B[*a*]P: Benzo[*a*]pyrene

B[*b*]F: Benzo[*b*]fluoranthene

B[*e*]P: Benzo[*e*]pyrene

B[*ghi*]P: Benzo[*ghi*]perylene

B[*k*]F: Benzo[*k*]fluoranthene

BSA: Bovine serum albumin

CDNB: 1- chloro-2,4-dinitrobenzene

Chr: Chrysene

CYP: Cytochrome P450

D[*ah*]A: Dibenz[*a,h*]anthracene

DEPC: Diethylpyrocarbonate

Fle: Fluorene

Flu: Fluoranthene

G-6-P: Glucose-6-phosphate

G-6-PDH: Glucose-6-phosphate dehydrogenase

GSH: Glutathione reduced form

GST: Glutathione-S-transferases

EROD: Ethoxyresorufin O-deethylation

HPLC: High performance liquid chromatography

Km: Michaelis constant

MROD: Methoxyresorufin O-demethylation

Nap: Naphthalene

PAHs: Polycyclic aromatic hydrocarbons

Phe: Phenanthrene

Pyr: Pyrene

qPCR: Quantitative real-time polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate polyacrylamide gel electrophoresis

2,3,7,8-TCDD: 2,3,7,8-tetrachlorodibenzo-*p*-dioxin

UGA: UDP-glucuronic acid

UGT: UDP-glucuronosyl transferases

Vmax: Maximum velocity

PREFACE

Meat-producing animals are exposed during their lifetime to a host of xenobiotics such as drugs, growth promoters and environmental contaminants. Many of these xenobiotics are endowed with sufficient lipophilicity to cross lipoid barriers and achieve substantial plasma and tissue concentrations. The vast majority of these chemicals do not benefit the host organism and are potentially detrimental to its survival and well-being, consequently, its immediate response is to eliminate them and protect itself from their deleterious effects.

Meat-producing animals find it extremely difficult to remove lipophilic chemicals from the body, so that for their elimination to be achieved, at first they must be metabolically converted to hydrophilic metabolites that can be readily excreted. For this reason they responded to these xenobiotics by developing a number of enzyme systems, residing largely in the endoplasmic reticulum and cytosolic fraction of the cell, that catalyse the metabolic pathways that lead to the production of hydrophilic metabolites. Undoubtedly, the most important enzyme systems responsible for the metabolism and elimination of xenobiotic chemicals are the cytochrome P450 (CYP) and phase II enzymes.

Cytochrome P450 comprises a ubiquitous system of heme-thiolate enzymes encountered in almost every organ, but with the highest concentration found in the liver, which consequently functions as the centre of xenobiotic metabolism. The cytochrome P450 is a superfamily, that is divided into a number of families (>40% similarity), which in turn are subdivided into subfamilies (>55% similarity) (Nelson et al., 1996), each of which may consist of one or more

enzymes. The CYP system is exploited by many organisms in the biosynthesis of endogenous compounds like steroids, vitamins, fatty acids, prostaglandins, leukotrienes, biogenic amines and pheromones. However, the majority of CYP substrates are xenobiotics such as drugs and environmental pollutants.

Three families (CYP1, CYP2 and CYP3) are mainly involved in biotransformation of xenobiotics. Among these, CYP1A1 which is probably the most inducible isoform, being induced by planar compounds in the liver and extrahepatic tissues of animals and humans. Its contribution to drug metabolism is limited, but it is undoubtedly the dominant CYP in the bioactivation of chemicals, including most major classes of carcinogens. Especially, CYP1A1 is believed to be responsible for the activation of more than 90% of known carcinogenic chemicals (Ioannides, 2006). The expression level of CYP1A enzyme in different animal tissues is used as a biomarker of environmental pollution because the concentration of the enzyme tends to increase on chemical exposure (Ioannides, 2006). Nevertheless, limited efforts have been devoted into defining of CYP1A expression and activity in the meat-producing animals.

Important phase II detoxification or drug-metabolizing enzymes are UDP glucuronosyltransferases (UGTs) and glutathione-S-transferases (GSTs). UGTs catalyse the conjugation of exogenous and endogenous, mainly lipophilic compounds with glucuronic acid, while GSTs catalyse the reaction of glutathione with mainly exogenous electrophiles and endogenous products of oxidative stress. Some overlap in substrate specificity may occur between these detoxification enzymes. Glucuronidation or conjugation with glutathione in general results in less biologically active molecules and enhances the water solubility of the conjugated products, which facilitates excretion from the body via bile or urine (Van der Logt et

al., 2003). However, the importance of phase II enzymes in the bioinactivation of promutagens and procarcinogens in the meat producing animals is less informed.

In this thesis, I clarified the biological defense systems to xenobiotics in the meat-producing animals as following:

In chapter I, to investigate the expression and distribution of xenobiotic-metabolizing enzymes, I molecularly screened the tissue-specific mRNA expression of various CYPs and phase II enzymes in cattle, the most important source for animal-derived food all over the world.

In chapter II, I studied the CYP 1A dependent activities in three meat-producing animals (deer, cattle and horses) compared to the rat as a reference species because of the importance of CYP1A as a biomarker for pre-slaughter exposure to xenobiotics. I chose deer and horse beside cattle as horses are used as source for meat in some Asian and Latin American countries, while deer is representing an on growing source of the exotic meat in different localities worldwide. As a first step, I studied the constitutive inter-species difference in CYP1A-dependent activities among this group of animals. Moreover, I analyzed the different kinetic parameters of CYP1A-dependent ethoxyresorufin O-deethylase (EROD) activity in order to investigate whether this inter-species difference is due to genetic bases or due to exposure to some xenobiotics.

In chapter III, I extended my study to investigate the inter-species difference in the response to a polycyclic aromatic hydrocarbon, benzo[*a*]pyrene (B[*a*]P), an ideal promutagenic and procarcinogenic xenobiotic. Also I confirmed that these activities are catalyzed by CYP1A subfamily. Moreover, I studied the mechanism of defense of these animals against the mutagenic activity of the B[*a*]P.

In chapter IV, I tried to explain that the accumulated carotenoids are contributing in the regulation mechanisms of the inter-species difference in CYP1A expression and dependent activities.

Because of the importance of camel species as food-producing animal in many African and Asian countries especially in Egypt so, I directed chapter V to study the cloning and expression of CYP1A1 in the various tissues of camel.

Chapter I

High expression of the mRNA of cytochrome P450 and phase II enzymes in the lung and kidney tissues of cattle

Introduction

The mammalian cytochrome P450 (CYP) superfamily is divided into a number of families, which in turn are divided into subfamilies, each of which consists of one or more enzymes. These enzymes metabolize a wide range of endogenous and exogenous xenobiotic compounds, resulting in either the activation or detoxification of the xenobiotics depending on the enzyme involved (Ioannides, 2006). Thus, the response of the body to physiological substrates, therapeutic drugs, carcinogens and other toxicants and pollutants can be greatly influenced by the differential expression of cytochrome P450 enzymes in different tissues (Guengerich, 1997).

In many species, the liver shows the highest expression of these enzymes and other phase II enzymes, but these enzymes are also expressed in extrahepatic tissues such as kidney, intestine, lung and tongue (Raza et al., 1998). It is recently reported the unique expression pattern of CYP1A1, in particular in ungulates, the high expression and the distribution of CYP1A1 mRNA in tongue are markedly different from the CYP distribution pattern in rats or

other laboratory animals (Nebbia et al., 2003; Takiguchi et al., 2010). These results led us to investigate the distribution of CYP and xenobiotic metabolizing enzymes in ungulates.

Cattle are, economically, one of the most important veterinary species worldwide. However, few studies have investigated the expression of different CYP isoforms and phase II conjugating enzymes in this species. Food-producing animals like cattle are often exposed to pesticides, pollutants and drugs, which are potentially harmful to the animal itself and also to humans, if animal tissues containing high levels of harmful residues are consumed (Giantin et al., 2008). Consequently, drug metabolism studies performed in these animals are important for the evaluation of consumer risk. Despite these obvious toxicological implications, our understanding of CYP and phase II conjugating systems in the liver and other tissues of cattle is limited (Nebbia et al., 2003). The most common approach to defining CYP composition in the hepatic and extra-hepatic tissues of animals is to use diagnostic probes and antibodies which are raised to individual CYP proteins. Most studies have used rat and human as the reference species, simply because they have been extensively studied and antibodies are commercially available. However, extrapolation of data obtained using rat or human probes or antibodies with the tissues of food-producing animals such as cattle, remains a difficult task because of the well-established species differences in the activity, expression and regulation of CYP proteins and phase II conjugating enzymes (Ioannides, 2006). On the contrary, bovine-specific primer pairs were designed for P450s and phase II enzyme isoform mRNA to be used in the relative quantification by means of quantitative real-time polymerase chain reaction (qPCR). In fact, the entire bovine genome has been recently sequenced but a definitive nomenclature for bovine P450s as well as phase II enzymes, is still actually lacking. For this reason, in the present study, bovine drug metabolizing

enzymes sequences were identified with the name of the human sequence sharing the highest percentage of identity, based on protein sequence alignments, followed by the suffix-like.

Thus, the objective of this chapter was to screen the expression pattern of various forms of CYP, UDP glucuronosyl transferase (UGT) and glutathione-S-transferase (GST) in cattle tissues using qPCR method. This may be a useful tool to help us in understanding the contributions of the extra-hepatic tissues in the xenobiotics metabolism. It also may reflect the pre-slaughter exposure to some xenobiotics which subsequently has a direct impact on the consumers risk evaluation.

Materials and methods

Chemicals and reagents

All test reagents used were of reagent grade including those described below. TRI reagent was purchased from Sigma (St Louis, MO, USA). Oligo(dT) primer, RT-buffer and ReverTra Ace were purchased from TOYOBO (Osaka, Japan). Primer sets were purchased from Invitrogen (Carlsbad, CA, USA). Ex Taq Polymerase was purchased from TaKaRa (Tokyo, Japan) All other reagents were analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries, Tokyo, Japan.

Animals

All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. Samples from liver, mammary gland, lungs, spleen, kidney cortex, heart, masseter muscle and tongue were collected from five adult female Holstein cattle (*Bos taurus*) from a Hokkaido University cattle farm. These cattle aged four to five years (4.67±0.58 years old) and were non-pregnant and non-lactating. The samples were excised immediately after slaughter and were transferred to liquid nitrogen and then were kept frozen at -80°C till use. These cattle had been reared on grass feed with no medical history for at least one month prior to sacrifice.

RNA extraction

Total RNA was prepared from each tissue by the single-step method (Chomczynski and Sacchi, 1987), using TRI reagent. from Sigma Chemical Co. (St. Louis, MO, USA). The concentration and purity of the RNA was determined spectrophotometrically at 260 and 280 nm, respectively.

cDNA synthesis

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 70ºC 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 Diethylpyrocarbonate (DEPC) water and 2 µL of reverse transcriptase (ReverTra Ace). The mixture was then reincubated in the thermal cycler at 30ºC for 10 min, 42ºC for 1h and 90ºC for 10 min to prepare the cDNA.

Quantitative real-time polymerase chain reaction (qPCR)

qPCR was performed to analyze the mRNA levels of cattle CYP1A1-like, 1A2-like, 2B6-like, 2C9-like, 2E1-like, 3A4-like, UGT1A1-like, GSTA1-like and β-actin-like, which was used as an endogenous control as it was equally expressed in all examined tissues, 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), according to the manufacturer's instructions. The primer sets used have been described previously (Giantin et al., 2008), and are shown in Table 1. The PCR reaction mixture was prepared with 1× Master Mix reagents (FINNZYMES Oy), 300 nM of each primer, 500 ng cDNA and 1× ROX reference dye in 1 µL

of RNase-free water, the mixture was made up to a final volume of 20 μL with RNase-free water. The reaction cycle comprised an initial holding stage at 95°C for 15 min, then 40 cycles of: denaturation at 95°C, annealing at an appropriate temperature (as described in Table 1) for 1 min, and extension at 72°C for 30 s. Melting curve analysis and agarose gel electrophoresis confirmed the amplification of a single amplicon of the expected size as well as the absence of primer dimers and genomic DNA amplification. Each measurement was performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of β-actin and was calculated relative to the corresponding gene expression in the liver for each animal individually. The tissue distribution of mRNA expression of each gene was expressed as the mean ± standard deviation (SD) of five animals.

Statistical analysis

Statistical significance was evaluated using the Tukey–Kramer Honestly Significant Difference test (JMP statistical package, SAS Institute, Cary, NC, USA). A p value <0.05 was considered to be significant.

Target	Primer sequence	AT	Amplicon size
CYP1A1-like (XM_588298)	F: 5'-GACCTGAATCAGAGGTTCTACGTCT-3' R: 5'-CCGGATGTGACCCTTCTCAA-3'	60°C	81
CYP1A2-like (XM_591450)	F: 5'-ACCATGACCCGAAGCTGTG-3' R: 5'-CAATGGTGGTGCCATCAGAC-3'	60°C	78
CYP2B6-like (NM_001075173)	F: 5'-GCGGACCTCATCCCCATT-3' R: 5'-GTGCCCTTGGGAAGGATGT-3'	60°C	80
CYP2C9-like (XM_612374)	F: 5'-TCCCTGGACATGAACAACCC-3' R: 5'-TTGTGCTTTTCCTGTTCCATCTT-3'	61°C	71
CYP2E1-like (NM_174530)	F: 5'-ACCCGGAGGTTGAAGAGAAAC-3' R: 5'-GCCCAATCACCCTGTCAATTT-3'	60°C	51
CYP3A4-like (NM_174531)	F: 5'-GCCAGAGCCCGAGGAGTT-3' R: 5'-GCAGGTAGACGTAAGGATTTATGCT-3'	60°C	77
UGT1A1-like (DQ115935)	F: 5'-ACCATCCTACGTGCCCAGG-3' R: 5'-TGTTCTTCACCCGCTGCAG-3'	62°C	71
GSTA1-like (NM_001078149)	F: 5'-TTCCCTCTGCTAAAGGCCCTA-3' R: 5'-CTTCCTCTGGCTGCCAGG-3'	60°C	84
β-Actin-like (NM_173979)	F: 5'-GTCGACACCGCAACCAGTT-3' R: 5'-AAGCCGGCCTTGCACAT-3'	61°C	85

Table 1: Primer pairs used for qPCR amplification of each target gene. Included in the table are the primer sequences, accession numbers of the genes, annealing temperatures (AT) and the lengths (base pairs, bp) of the PCR products.

Results

mRNA expression of cytochrome P450s in the different tissues of the cattle

In this chapter, I analyzed the mRNA expression of cytochrome P450s in the different tissues of the cattle. The expression of CYP1A subfamily, which is divided into CYP1A1 and CYP1A2, was examined. CYP1A1-like mRNA was expressed in all tissues examined both in liver and outside liver . The highest expression level was recorded in the kidneys compared to the other tissues examined (Fig. 1A). A relatively higher expression of CYP1A1-like mRNA was observed in liver, tongue, lung, heart and mammary gland (Fig. 1A). The lowest CYP1A1-like mRNA was recorded in both spleen and muscle (Fig. 1A). CYP1A2-like mRNA was expressed mainly in the liver while other examined tissues showed very small expression pattern compared with liver (Fig. 1B).

The tissue-specific expression of CYP2 family-like mRNAs, CYP2B6, CYP2C9 and CYP2E1-like mRNAs was examined in the different tissues of cattle. CYP2B6-like mRNA was expressed in all tissues examined both in liver and outside liver. The lung and liver showed significantly higher expression than other examined tissues (Fig. 1C). CYP2C9-like mRNA was expressed mainly in the liver, followed by the kidney. Other examined tissues either did not show any expression or showed very small expression (Fig. 1D). CYP2E1- and CYP3A4-like mRNAs were expressed in liver only as clear in figures 1E and F, respectively.

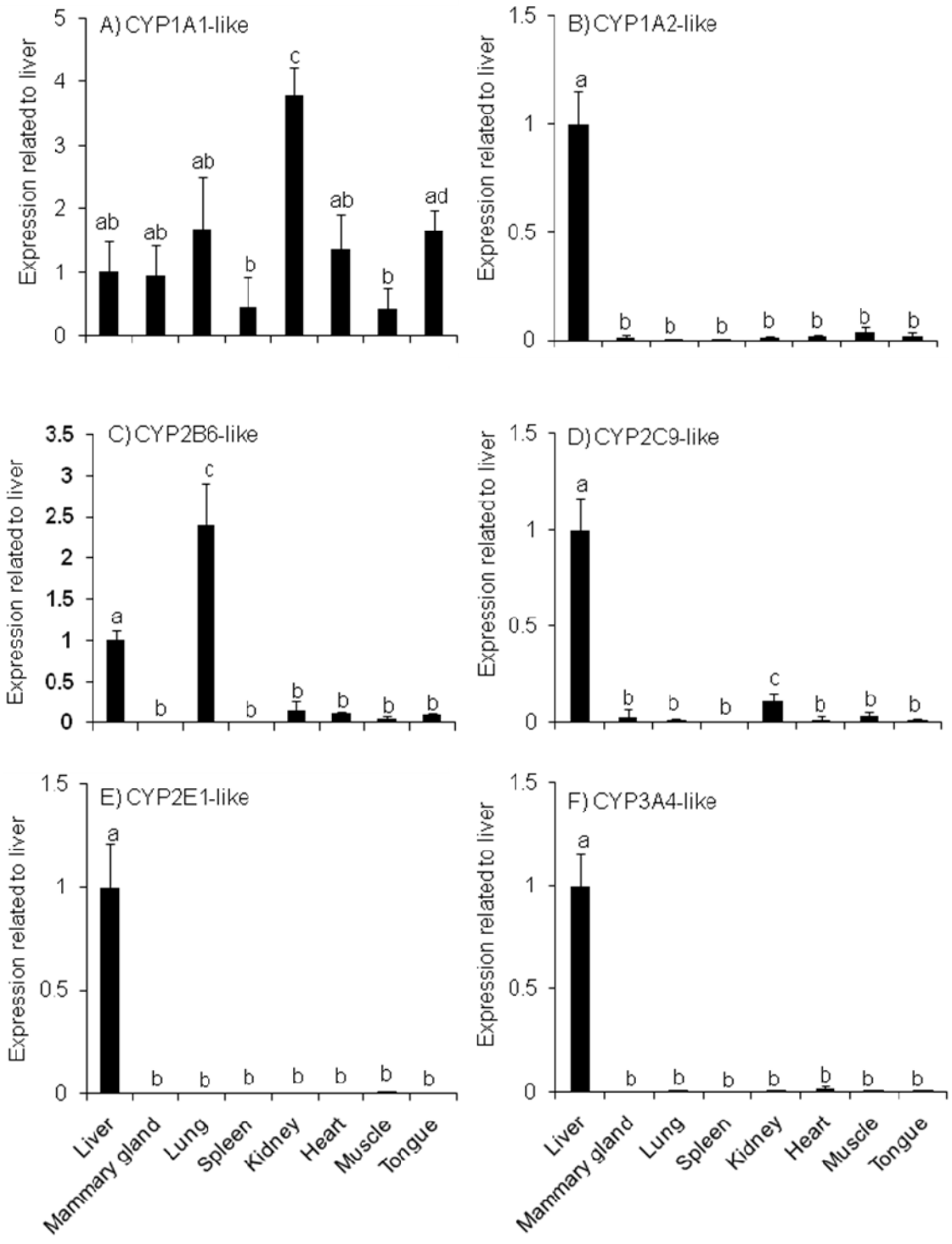
A) CYP1A1-like
B) CYP1A2-like
C) CYP2B6-like
D) CYP2C9-like
E) CYP2E1-like
F) CYP3A4-like
Expression related to liver
Liver
Mammary gland
Lung
Spleen
Kidney
Heart
Muscle
Tongue

Fig. 1. Expression of different cytochrome P450 isoforms in cattle offal.

The mRNA expression levels of different cytochrome P450 isoforms in the different tissues of cattle compared with the levels in the liver using qPCR analysis of: A) CYP1A1-like, B) CYP1A2-like, C) CYP2B6-like, D) CYP2C9-like, E) CYP2E1-like, and F) CYP3A4-like genes. PCR amplification of the cDNA samples was carried out as described in the text. Each measurement was performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of β-actin and was calculated relative to that of the liver. The value relating to expression in the liver was adjusted to 1. The tissue distribution of mRNA expression of each gene was expressed as the mean ± standard deviation (SD) of five animals. Identical letters indicate no significant difference. $P<0.05$.

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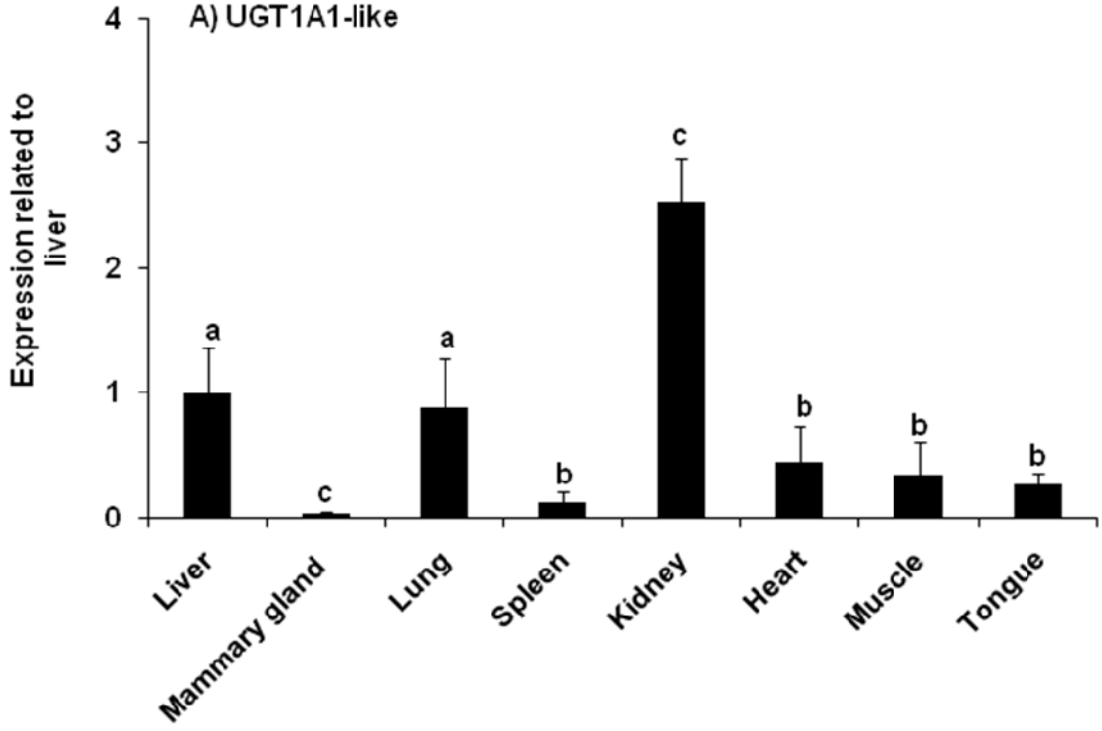
mRNA expression of phase II enzymes in the different tissues of the cattle

Phase II enzymes were represented in two major enzymes, UDP glucuronosyl transferase 1A1 (UGT1A1) and glutathione-S-transferase (GSTA1). The tissue-specific expression of UGT1A1 and GSTA1-like mRNAs was examined in all tissues compared with liver. UGT1A1-like mRNA was expressed in all tissues. Kidney showed the highest expression followed by liver and lung (Fig. 2A). Other examined tissues showed similar expression pattern for UGT1A1-like mRNA, but higher than the mammary gland (Fig. 2A). Likely, GSTA1-like mRNA was expressed in all tissues of cattle. The highest expression was observed in the renal tissue followed by liver and muscle. Other examined tissues showed variable expression pattern as shown in figure 2B.


A) UGT1A1-like
Expression related to liver
4
3
2
1
0
a
c
a
b
c
b
b
b
Liver
Mammary gland
Lung
Spleen
Kidney
Heart
Muscle
Tongue

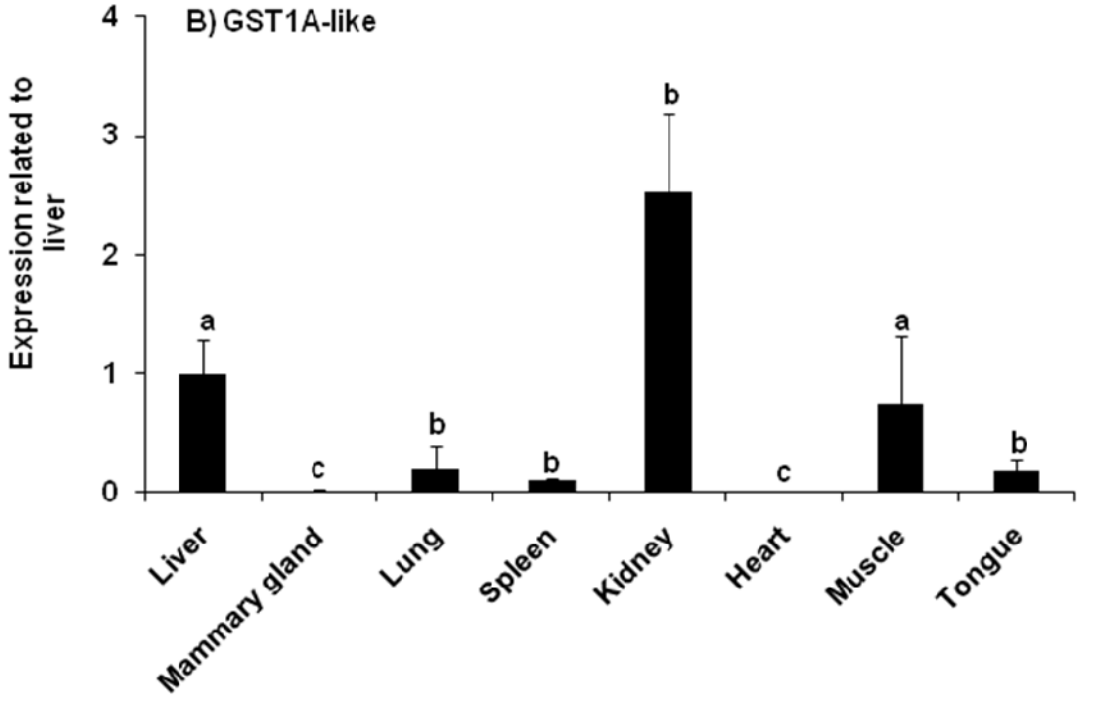

B) GST1A-like
Expression related to liver
4
3
2
1
0
a
c
b
b
b
c
a
b
Liver
Mammary gland
Lung
Spleen
Kidney
Heart
Muscle
Tongue

Fig. 2. Expression of different phase II enzymes in cattle offal

The mRNA expression levels of different phase II enzymes in the different tissues of cattle compared with the levels in the liver using qPCR analysis of: A) UGT1A1-like, and B) GSTA1-like genes. PCR amplification of the cDNA samples was carried out as described in the text. Each measurement was performed in duplicate and repeated three times. The expression of each gene was normalized to the expression of β-actin and was calculated relative to that of the liver. The value relating to expression in the liver was adjusted to 1. The tissue distribution of mRNA expression of each gene was expressed as the mean ± standard deviation (SD) of five animals. Identical letters indicate no significant difference. $P<0.05$.

Discussion

In mammals, CYP-associated expression and activity are found in all tissues, but predominantly occur in the liver in most species. In extrahepatic tissues, CYP enzymes involved in the metabolism of xenobiotics are mainly concentrated in those organs through which foreign substances pass when entering the body such as lung, intestine and skin (Guengerich, 1997). Tissue-specific expression of CYP isoforms and phase II conjugating enzymes has been extensively studied in humans and laboratory animals but has received little attention in food-producing animals, like cattle. Moreover, the expression level of different CYP isoforms and phase II enzymes is considered as a good biomarker for pre-slaughter exposure to different xenobiotics because the concentration of these enzymes tends to increase on chemical exposure (Fujita et al., 2001). The use of different CYPs and phase II enzyme induction as an assessment technique has increased in recent years. This is mainly due to the optimization of protocols for rapid and relatively inexpensive of its expression and activity measurements (Okey, 1990). Thus, in this chapter, I investigated the mRNA expression pattern of different CYP proteins and conjugating enzymes in cattle.

The CYP1A subfamily comprises two isoforms, 1A1 and 1A2. It is probably the most inducible CYP subfamily, being induced by planar compounds in the liver and extrahepatic tissues of animals and humans. CYP1A1 is mainly expressed extrahepatically, its hepatic level is very low in rats and humans (Ioannides and Parke, 1990). Surprisingly, CYP1A1-like mRNA is expressed in all tissues examined including the liver. The highest expression level was recorded in the kidney of cattle. That high expression level in the kidney and the expression of CYP1A1 in the liver differed from the findings reported in rats. High expression of CYP1A1 in liver and

kidneys strongly suggests that CYP1A1 is induced either by environmental pollutants, such as polycyclic aromatic hydrocarbons, or by phytochemicals (Ioannides, 2002). In line with this result, CYP1A1 protein was highly expressed in bovine liver (Sivapathasundaram et al., 2001). In a previous report, the cattle liver also showed higher CYP1A1-dependent ethoxyresorufin O-deethylase (EROD) activity compared with rat liver (Darwish et al., 2010b). Interestingly, other examined tissues such as the lung, tongue and mammary glands, showed similar expression pattern to that observed in the liver. The high expression of CYP1A1-like mRNA in the lungs and tongue corresponds to that reported by Darwish et al. (2010a), who observed higher expression for CYP1A1-like mRNA in the lungs and tongue of camel. Takiguchi et al. (2010) also observed high CYP1A1 mRNA and protein expression in the tongue of the rat. The relatively high expression of CYP1A-like mRNA in the mammary glands may be correlated to the occurrence of a remarkable rate of aflatoxin B1 hydroxylation to aflatoxin M1 in a clonal cell line established from primary bovine mammary epithelial cells as mentioned by Caruso et al. (2009). Moreover, it was reported that the mRNA and protein expression of CYP1A and CYP1A2 were significantly induced by cow milk-based formula in HepG2 cells (Xu et al., 2005). Also, there were much concerns about the contamination of the human breast milk with carcinogenic and mutagenic environmental pollutants, such as halogenated aromatic hydrocarbons (e.g., dioxin and polychlorinated biphenyls (PCB)) and polycyclic aromatic hydrocarbons (PAH) (Hooper and McDonald, 2000). Although, the mammary gland samples were collected from non-pregnant and non-lactating cows but the role of the residual milk or accumulated pollutants cannot be ignored in the induction of CYP1A1 in this tissue.

CYP1A2, which is involved in the metabolism of caffeine and heterocyclic amines (Ioannides, 2002), was expressed mainly in the liver. Unlike the results with CYP1A1, the CYP1A2 results agree with those reported in rats and humans (Ioannides, 2006).

The induction of CYP1A mRNAs in the different tissues of the cattle is mainly mediated though the binding of xenobiotics to a cytosolic aryl hydrocarbon receptor (AhR). AhR ligands usually have esoteric configurations and may be similar in structure to 2,3,7,8-teterachlorodibenzo-p-dioxin (2,3,7,8-TCDD), one of the typical CYP1A inducers (Nebert et al., 1993).

The CYP2 family is the largest mammalian CYP family, comprising a number of distinct subfamilies that markedly differ in their substrate specificity. In rats, CYP2B is mainly expressed hepatically (Pascussi et al., 2003). However, interestingly in cattle, CYP2B6-like mRNA is highly expressed in the lung compared with the other tissues examined. This result can be explained by the fact that this isoform participates in the bioactivation of nitrosamines and the carcinogenic mycotoxin aflatoxin B1 (Chang et al., 1993). The CYP2C subfamily is composed of at least four enzymes in humans and is responsible for the metabolism of many major drugs, such as rifampicin (Ioannides, 2006). CYP2C9-like mRNA was found to be expressed mainly in the liver and kidney of cattle, confirming a previous report of CYP2C9 expression in the liver of cattle (Grasso et al., 2005) and corresponds with Pegolo et al. (2010), who demonstrated CYP2C9-like activities in the bovine liver microsomes. The CYP2E subfamily consists of a single enzyme CYP2E1, which is induced after exposure to small molecular weight xenobiotics, such as acetone and alcohol. CYP2E1-like mRNA is expressed only in the liver in cattle, similar to the expression in rats and humans (Grasso et al., 2005). This result corresponds with

Szotáková et al. (2004) who observed a protein cross reacting with anti-human CYP2E1 IgG in the bovine liver. The CYP3 family is the most active contributor in drug metabolism. CYP3A4-like mRNA was expressed only in cattle liver. Cattle liver microsomes catalyze the dealkylation of CYP3A-substrates, such as erythromycin (Grasso et al., 2005). This result corresponds with Szotáková et al. (2004) who observed a protein cross reacting with anti-human CYP3A4 antibody in the bovine liver.

With regards to phase II conjugating enzymes, UGTs catalyze the conjugation of exogenous and endogenous lipophilic compounds (mainly) with glucuronic acid. UGTs comprise two families, UGT1 and UGT2. UGT1 enzymes mainly catalyze glucuronidation of exogenous agents such as drugs, pesticides and benzo[a]pyrene (Van der Logt et al., 2003). UGT1A1-like mRNA was expressed in all tissues examined, except for the mammary glands. Many UGT1 isoenzymes have also been expressed in extrahepatic tissues, including kidney, stomach, small intestine, colon, tongue and many other tissues in rats and mice (Buckley and Klaassen, 2007; Takiguchi et al., 2010). However, surprisingly in cattle, kidney showed the highest expression pattern for UGT1A1 compared with the expression in liver. Unlike rats and mice, cattle were unique in the high level of expression of UGT1A1 in lung, suggesting that glucuronidation of some inhaled pollutants may initiate in the lungs.

GST, similar to UGT, is a major phase II biotransformation enzyme, which conjugates hydrophilic moieties to hydrophobic substrates, thereby affecting their inactivation and clearance. To date, seven GST classes have been described in mammals. Of these, GST alpha (A) contributes significantly to the biotransformation of promutagens and procarcinogens (Giantin et al., 2008). We report here that GSTA1-like mRNA was expressed in all cattle tissues examined,

except for heart and mammary glands. In line with UGT1A1-like mRNA expression, the highest GSTA1-like mRNA expression was reported in the kidney. Accordingly, rat kidney showed higher GSTA1-dependent CDNB conjugative activity compared to lung, brain and respiratory epithelium (Ben-Arie et al., 1993). In mice liver, GSTA1 mRNA showed very low expression (Knight et al., 2007). In contrast to the findings in mice, cattle liver expressed lower levels of GSTA1 mRNA than kidney, but higher expression levels than other tissues.

The high expression pattern of phase II enzymes, UGT1A1 and GSTA1 mRNAs in the liver, lung and kidney which showed also high expression for different CYP isoforms mRNAs is suggesting that phase II enzymes play a great role in producing a state of balance between the bioactivation and detoxification of xenobiotics in the meat-producing animals, particularly cattle.

In conclusion, to the best of our knowledge, this is the first report to study the tissue-specific expression of various CYP isoforms, UGT1A1 and GSTA1 mRNAs in cattle. My findings suggest that liver, lung and kidney in cattle are the major organs contributing to xenobiotic metabolism, and thus, this may have toxicological implications for public health.

Chapter II

Cytochrome P450 1A -dependent activities in the meat-producing animals

Introduction

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 in mammals. 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 (Elskus et al., 1999; Gonzalez and Kimura, 2003; Teraoka et al., 2003; Goldestone and Stegeman, 2006).

In mammals, the CYP1A subfamily has two isoforms (CYP1A1 and CYP1A2), which have highly homologous amino acid sequences (more than 70% identity) (Kimura et al., 1986; Omiecinski et al., 1990). 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 (Ioannides and Parke, 1990).

Comparisons of the catalytic selectivity of individual CYP enzymes have been published for human and laboratory animals such as rodent species, dogs and monkeys (Weaver et al.,

1994; Guengerich, 1997; Shimada et al., 1997; Anzenbacher et al., 1998; Ioannides, 2006). However, only limited knowledge on hepatic CYP enzymes in other animal species, especially in ungulates like deer, cattle and horses, is currently available (Sivapathasundaram et al., 2001; Machala et al., 2003; Nebbia et al., 2003; Sivapathasundaram et al., 2003a). 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 (Nebbia et al., 2003). 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 (Juskevich, 1987).

In this chapter, I attempted to investigate more about the metabolic activity of CYP1A in the meat-producing animals such as 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 kinetics 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, USA). 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, USA). 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).

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 female animals. Holstein cattle (*Bos taurus*) samples were collected from a Hokkaido University cattle farm at the age of four to five 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 four to six years (5.33 ± 1.15 year old). Ezo sika deer (*Cervus nippon 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 (1964). Briefly, livers were minced and homogenized in 3 volumes of ice-cold 1.15% KCl solution using a cap 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. The suspension was divided into 1.5-ml 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. (1951) 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 $91 mM^{-1} cm^{-1}$ (Omura and Sato, 1964).

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. (1985) 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 0.1M 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 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/μL, 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 EROD and MROD assays to the microsomes preincubated with antibody or nonimmune serum, as mentioned previously (Burke et al., 1985).

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 cap software Inc, La Jolla, CA, USA). Lineweaver-Burke 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 test using JMP (SAS Institute, Cary, NC, USA). A $p < 0.05$ 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 2). 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. 3A). 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. 3B).

Table 2. 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 ± 0.03^{a}	0.55 ± 0.02^{b}
Vmax (pmol/min/mg)	107.5 ± 10.5^{a}	241.2 ± 8.1^{b}	337.1 ± 2.4^{c}	918.2 ± 53.4^{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}	0.110 ± 0.004^{c}	0.150 ± 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 five animals from each species. Values with identical superscript letters are not significantly different from each other (p <0.05).

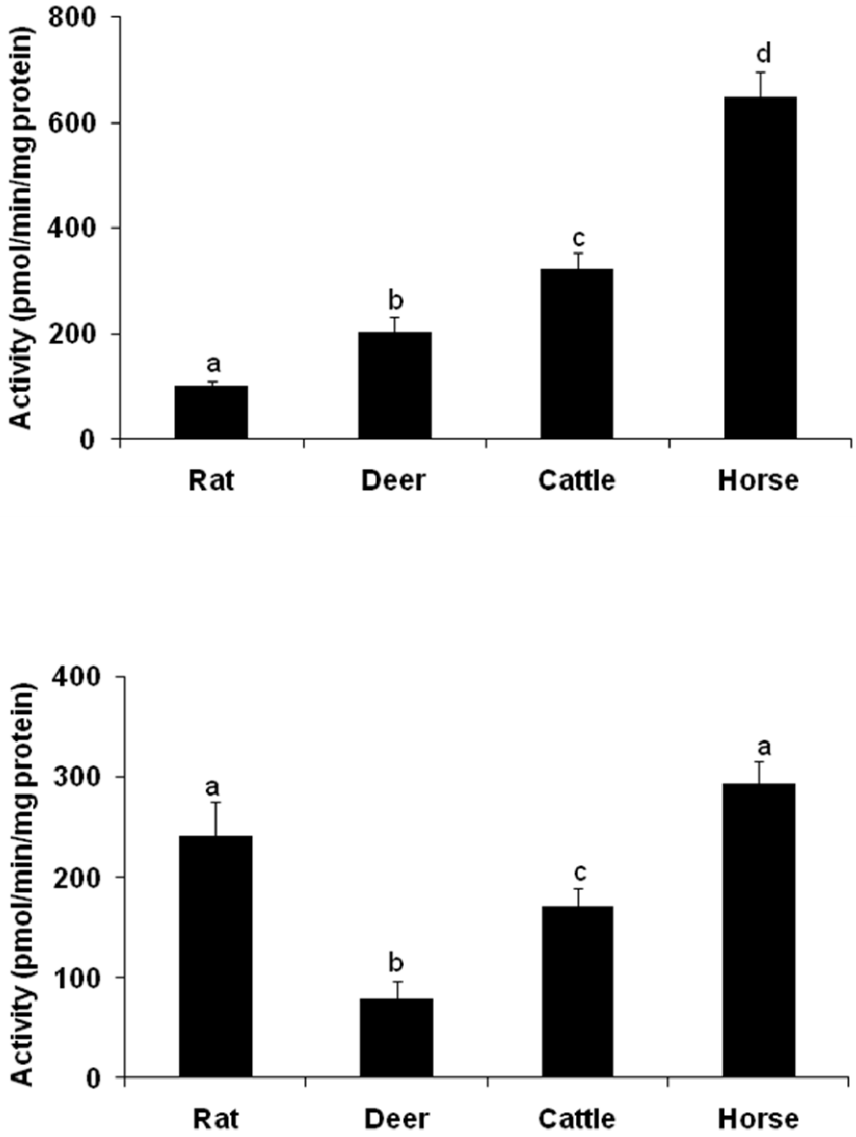


Fig. 3. CYP1A-dependent activities in liver microsomes of the deer, cattle and horses compared with those of the rats

EROD (A) and MROD (B) activities in the deer, cattle and horses compared with the rats using a substrate concentration of 20 μM for each. The data represent the means ± SD for five animals from each species. Values with identical superscript letters are not significantly different from each other (p <0.05).

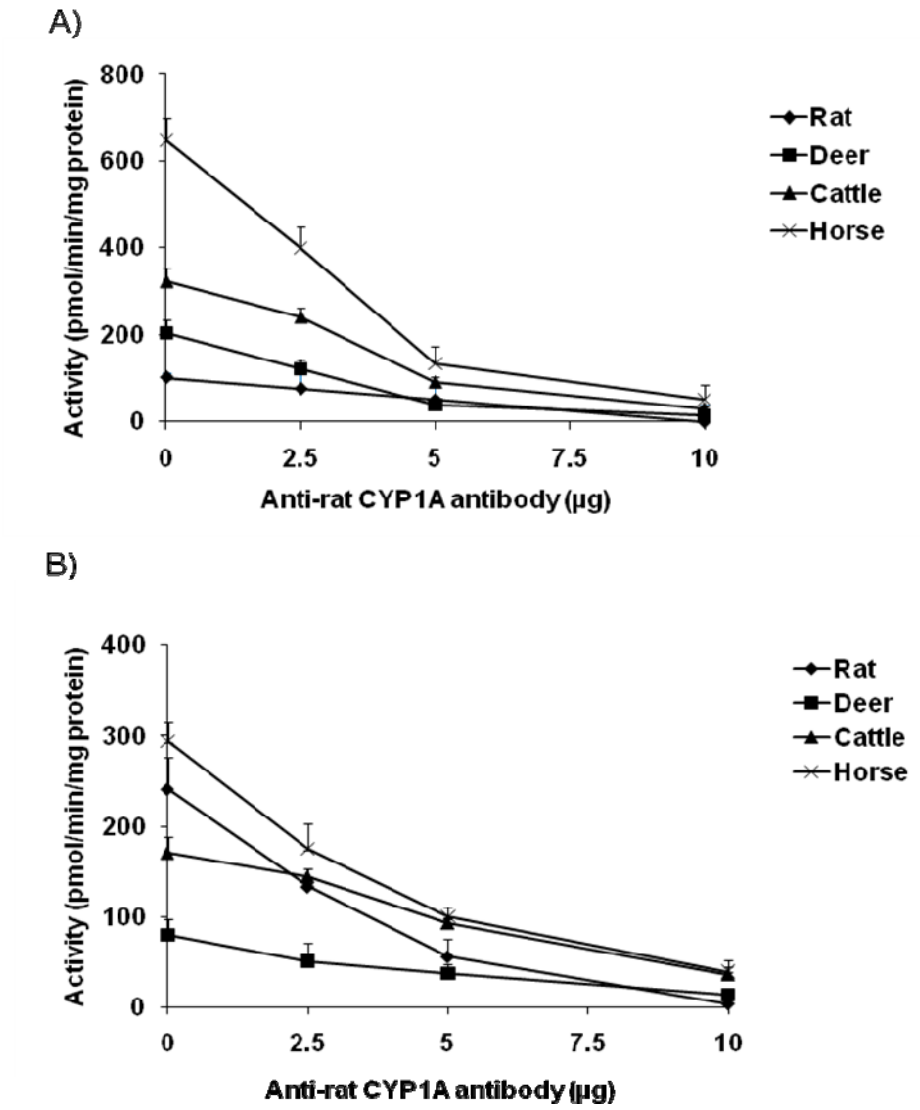


Fig. 4. Inhibition of EROD and MROD activities in liver microsomes of the deer, cattle and horses compared with those of the rats by anti-rat CYP1A1 antibody

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 μM) and different concentrations of the inhibitor, anti-rat CYP1A1 antibody (2.5, 5 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 five animal liver microsomes. Data at the concentrations of 2.5, 5 and 10 μg of the antibody are significant compared with the data at the concentration of 0 μg antibody in all animals (p <0.05).

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. 4).

Kinetic analysis of EROD activity

To determine the catalytic efficiency of CYP1A in the animal species examined in this study, I analyzed the kinetic parameters for EROD activity over a wide range of substrate concentrations ranging from 0.312 to 20 μM. The results showed that the 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 2). 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 Table 2 and Figure 5.

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. 6).

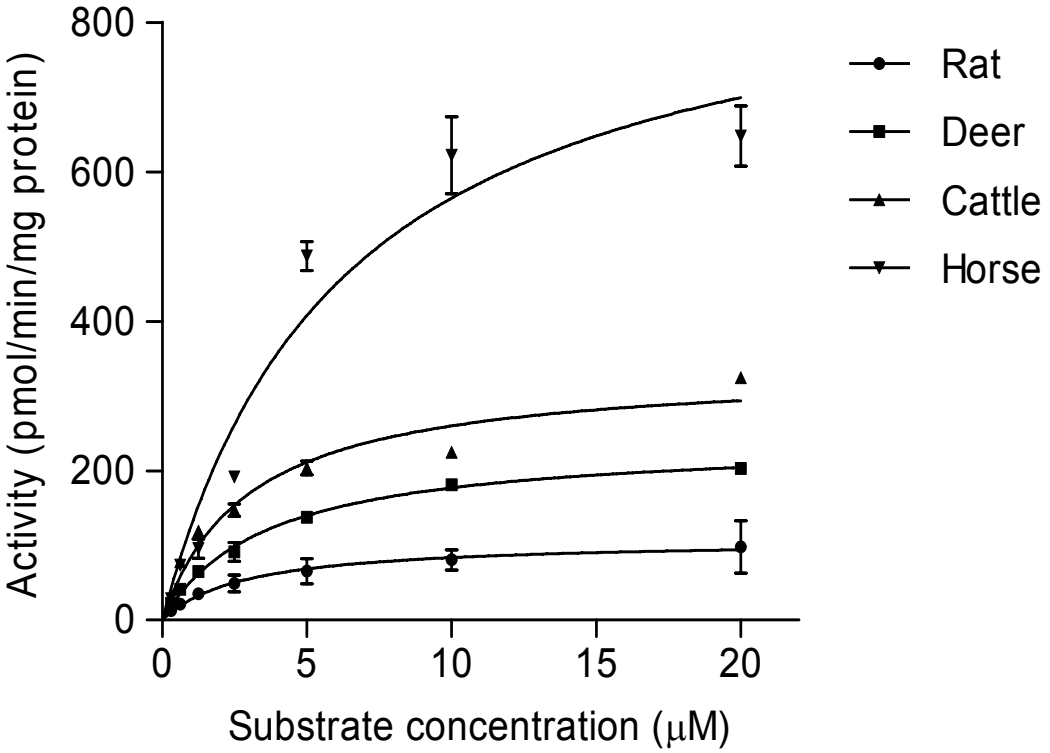


Fig. 5. Hyberbolic regression kinetic analysis for EROD activity in the deer, cattle and horses compared with the rats

Ethoxyresorufin O-deethylase activity was measured over substrate concentrations ranging from 0.312 to 20 μM. Data represent means of three experiments performed at different times using five animal liver microsomes.

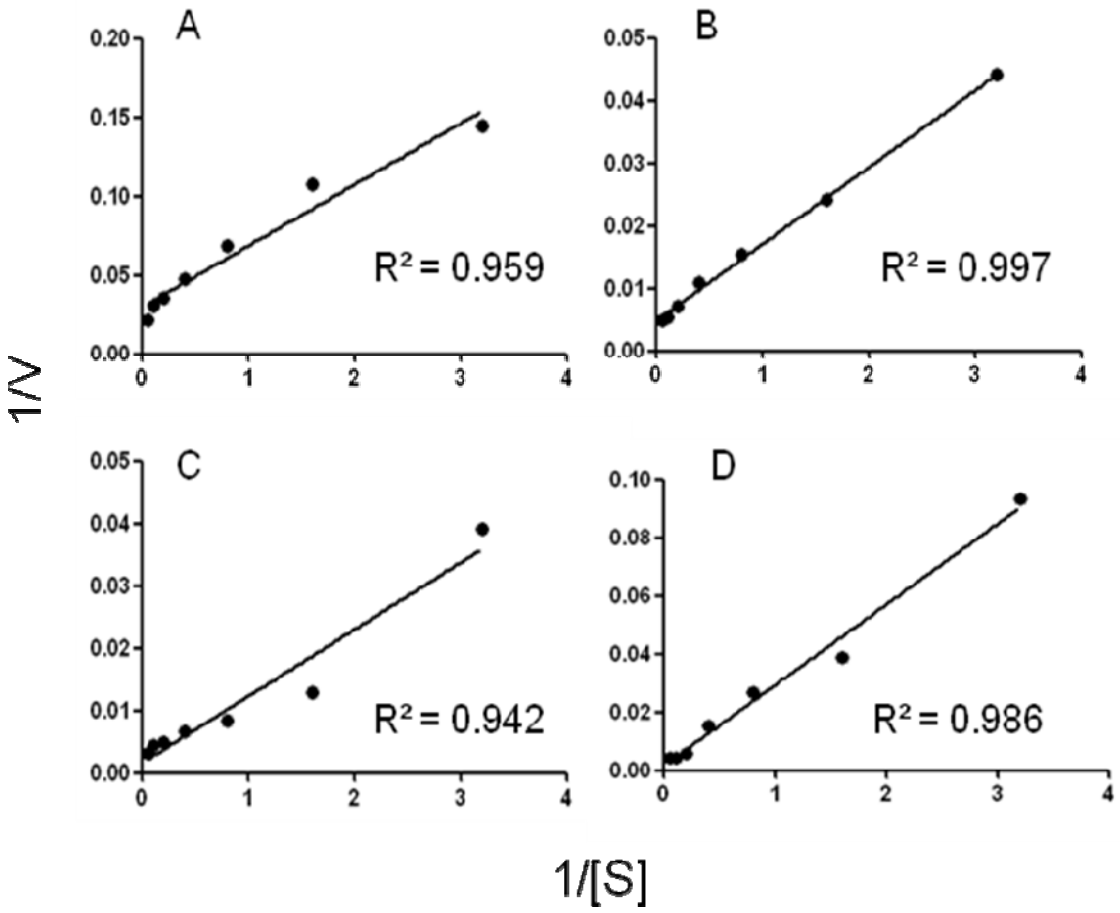


Fig. 6. Kinetic analysis of EROD activity using Lineweaver Burk plots in the deer, cattle and horses compared with the rats

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 μM. Data represent means of three experiments performed at different times using five animal liver microsomes.

Discussion

In the present chapter, I defined CYP1A-dependent activities in the meat-producing animals like cattle, deer and horses compared with rats using EROD and MROD assays (Ioannides, 1999; Sivapathasundaram et al., 2003a). 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 the evidence 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 (Burke et al., 1994). 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 I know, there is no information available on which CYP isoform is responsible for EROD and MROD activities in ungulates, including deer, cattle and horses. In the previous reports (Sivapathasundaram et al., 2001 and 2003a), 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 (Omiecinski et al., 1990; Ioannides, 2006). Therefore, I tried to confirm

my 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. I 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 rats. 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, I 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, followed by bovine and cervine microsomes, respectively. I 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. I 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.

Chapter III

Mutagenic activation and detoxification of benzo[*a*]pyrene *in vitro* by hepatic cytochrome P450 1A1 and phase II enzymes in the meat-producing animals

Introduction

Polycyclic aromatic hydrocarbons (PAHs) such as benzo[*a*]pyrene (B[*a*]P) are commonly formed by the incomplete combustion of organic matter (Baird et al., 2005; Shimada et al., 2007). A number of polycyclic aromatic hydrocarbons, including B[*a*]P, are mutagenic and carcinogenic, and are widely believed to make a substantial contribution to the overall burden of cancer in humans and animals (Phillips, 2002; Shoket, 1999). B[*a*]P requires metabolic activation by phase I enzymes, especially cytochrome P450 (CYP) 1A, prior to reaction with DNA, to exert its genotoxic effects (Phillips, 2005; Shimada, 2006; Volker et al., 2008).

Meat-producing animals like cattle, deer and horses are in danger of exposure to promutagenic and procarcinogenic polycyclic aromatic hydrocarbons such as B[*a*]P, and the principle route of exposure is via their diet (Phillips, 1999; Phillips, 2002). Moreover, results obtained by several researchers showed that these meat-producing animals have drastically higher CYP1A-dependent ethoxyresorufin-O-deethylase (EROD) activities than rats (Machala et al., 2003; Nebbia et al., 2003; Sivapathasundaram et al., 2001; Darwish et al., 2010a). This ungulate-specific phenomenon suggests the probability that these animals are exposed to large amounts of metabolically-activated mutagens and carcinogens. However, there is no clear

information about the mechanism of protection in meat-producing animals against the adverse effects of such exposure to these promutagens.

Living organisms have developed enzyme systems that metabolize xenobiotics to hydrophilic and readily excretable metabolites. Such metabolism usually proceeds through distinct stages: phase I metabolism introduces a functional group into the molecule, and phase II metabolism involves conjugation of the phase I metabolites through the functional group, with substrates such as glutathiones and glucuronic acids (Ioannides, 2002; Nebert and Dalton, 2006; Sivapathasundaram et al., 2003b) (Fig. 7). The phase II metabolism is catalyzed by a number of microsomal and cytosolic enzyme systems such as the UDP-glucuronosyl transferases (UGT), and glutathione-S-transferases (GST). These enzyme systems are very important to produce a state of equilibrium between the bioactivation and bioinactivation of foreign chemicals in the bodies of the living organisms (Sivapathasundaram et al., 2003b) (Fig. 7). Thus, in this chapter, I investigated the ability of the hepatic microsomes of cattle, deer and horses to mutagenically activate B[*a*]P compared to rats in a comparative way. In addition, the role of phase I and II enzymes in that mutagenic activation, and the mechanism of protection from this mutagenic activity were investigated in this group of animals. Rats were used as reference species in this study because the role of their phase I and II enzymes in the mutagenic bioactivation and detoxification of B[*a*]P is well documented (Fujita et al., 1988).

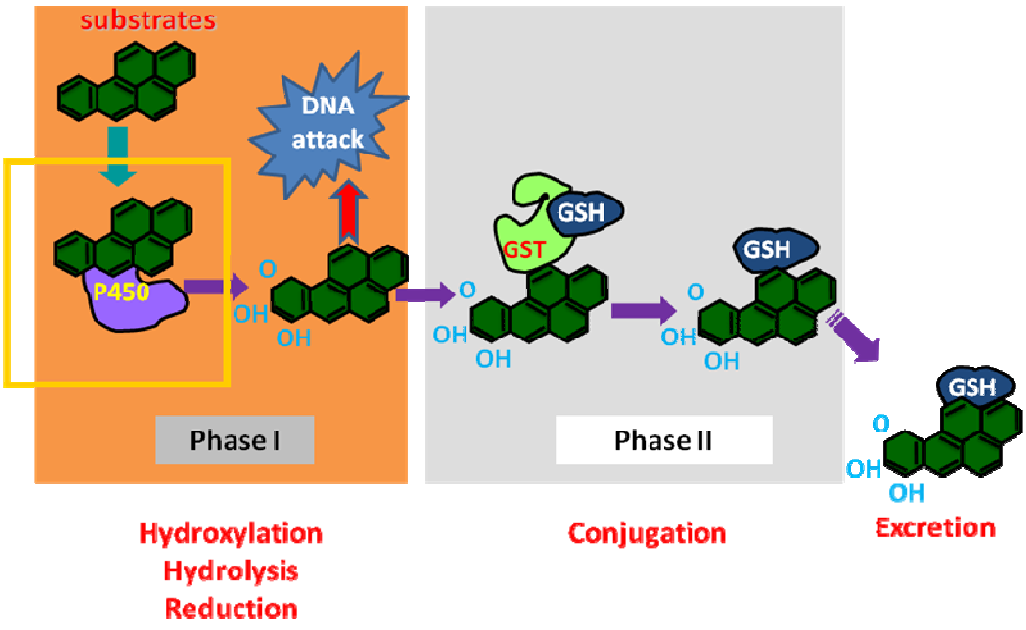


Fig. 7. Xenobiotics metabolic passway by phase I and phase II enzymes

Materials and methods

Chemicals and reagents

All test reagents used were of reagent grade. Benzo[*a*]pyrene, α-naphthoflavone, ρ-nitrophenol, 1-chloro-2,4-dinitrobenzene (CDNB), glutathione reduced form (GSH), UDP-glucuronic acid (UGA) and bovine serum albumin (BSA) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). NADPH, glucose-6-phosphate (G-6-P), glucose-6-phosphate dehydrogenase (G-6-PDH), and co-factor S9 were from Oriental Yeast (Tokyo, Japan). Polyclonal rabbit anti-rat CYP1A1, CYP3A2, and CYP2E1 antibodies (Daiichi Pure Chemical Co., Ltd., Tokyo, Japan), and rabbit IgG were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-rat CYP1A1 is reported to cross-react with the CYP1A2 of rat microsomes. All other reagents were analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries (Tokyo, Japan).

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 collected 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 sika deer (*Cervus nippon 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). 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 laboratory feed 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.

Preparation of liver microsomes

Liver microsomes from the animals were prepared by the method described by Omura and Sato (1964) with slight modifications. Livers were minced and homogenized in three volumes of ice-cold 1.15% KCl 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, divided among 1.5 ml tubes, snap-frozen in liquid nitrogen, and kept at -80°C until use. The protein concentrations of the microsomal, cytosolic, and S9 fractions were determined according to the method of Lowry et al. (1951) using bovine serum albumin (BSA) as the standard.

Ames mutagenicity assay

A mutagenicity assay was performed according to the method described by Ames et al. (1975) with slight modifications. Briefly, *Salmonella typhimurium* strain TA98, which is

sensitive to frameshift mutations, was used. One milliliter of the reaction mixture containing 10 mg of protein, 10 mM G-6-P, 1 or 10 μM B[*a*]P in dimethyl sulfoxide (Fujita et al., 1988), 1 mg co-factor S9 mixture. 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 20 min at 37°C, the reaction was terminated by adding of top agar (maintained at 45°C). The tubes were then immediately plated onto minimum glucose plates (2% glucose, 15% agar) in duplicate and incubated at 37°C for 48 h. Histidine-independent mutants were scored manually using a colony counter. Background colony formation was consistently between 28 and 35 colonies/plate.

Inhibition of mutagenic activation of B[a]P

To inhibit B[*a*]P mutagenic activation, α-naphthoflavone, a well-known chemical inhibitor of the CYP1 subfamily (Ioannides, 2006; Sivapathasundaram et al., 2003a), was added at concentrations of 2.5, 5, 10, or 20 μM to the reaction mixture using 10 μM B[*a*]P. Immunoinhibition was performed in this experiment using a rabbit anti-rat CYP1A1 antibody that recognizes both isoforms of the CYP1A subfamily. To investigate the contribution of other CYP subfamilies in this activity, anti-rat CYP3A2 and anti-rat CYP2E1 antibodies were used. The used anti-rat CYP antibodies were reported to cross-react with CYP subfamily proteins from other species (Sivapathasundaram et al., 2001; Nebbia et al., 2003). Antibodies were preincubated with microsomes in a concentration range of 2.5-10 μg/μL for 30 min based on the manufacturer's instructions and the method recommended by Fukuhara et al. (1999). The assays were subsequently completed as described previously with the use of 10 μM B[*a*]P. At the same

time, rabbit IgG was used as a negative control with the same concentrations as the antibody. The effect of the inhibitors on the bacterial growth was also examined.

Effect of phase II enzymes on mutagenic activation of B[a]P

To investigate the effect of phase II enzymes on the mutagenic activation of B[*a*]P, UGA, a co-factor for UGT, was added in different concentrations (0.2, 1, and 10 mM) or GSH (10, 20, and 30 mM) in 50 mM phosphate buffer (pH 7.5) to the reaction mixture using 10 μM B[*a*]P and incubated it at 37°C for 20 min prior to addition of the top agar as described by Fujita et al. (1988). In the case of GSH, the liver S9 fraction was used instead of liver microsomes because of the higher concentration of GST in the cytosol than in the microsomes. The effect of the co-factors on the bacterial cell viability was also examined.

UGT activity in meat-producing animals and rats

An assay was performed using ρ-nitrophenol as a substrate according to the method described by Fujita et al. (1988) with minor modifications. Briefly, the reaction mixture was prepared in an optical cuvette containing 0.5 mg/mL microsomal fraction, 0.02% Triton X-100, 5 mM $MgCl_2$, 0.25 mM ρ-nitrophenol, and 1.5 mM of UGA in 0.1 M Tris-HCl buffer (pH 7.0) in the final solution. The reaction was started by the addition of UGA. The decrease in absorbance at 400 nm was continuously measured using a Hitachi U3300 spectrophotometer. The reaction was linear with time for at least 2 min. Further linearity of the reaction was obtained by increasing UGA concentrations.

GST activity in meat-producing animals and rats

CDNB was used as a substrate to determine of GST activity, according to the method described by Habig et al. (1974) with minor modifications. In short, the reaction mixture contained final concentrations of 25 μg/mL cytosolic fraction, 0.5 mM CDNB, and 0.5 mM GSH in 0.1 M potassium phosphate buffer (pH 7.0). The mixture was preincubated at 37°C for 2 min, and the reaction was started by the addition of GSH. Incubation was carried out in a shaking water bath for 3 min and was terminated by the addition of 33% trichloroacetic acid. The mixture was centrifuged at 1500 x g for 10 min. The absorbance of the supernatant was determined at a wavelength of 340 nm. The same procedure was applied to the assay mixture without the cytosolic fraction to assess non-enzymatic conjugation. The amount of enzymatic CDNB-GSH conjugate formed was calculated by subtracting the non-enzymatic conjugate formation from the total conjugate formation using an extinction coefficient of 9.6 mM^{-1} cm^{-1} for the GSH conjugate of CDNB.

Statistical analysis

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

Results

Mutagenic activation of B[a]P by liver microsomes of meat-producing animals and rats

The liver microsomes of all animals had the ability to mutagenically activate B[*a*]P in the Ames mutagenicity assay. Horse CYP1A had the highest ability to produce revertant colonies through B[*a*]P hydroxylation among the species examined in the two concentrations examined. The mean values of the revertant colonies produced by horse, cattle, deer and rat liver microsomes were 71 ± 7, 48 ± 4, 49 ± 6 and 49 ± 2 at 1 μM B[a]P, and 138 ± 12, 100 ± 10, 70 ± 1 and 82 ± 13 at 10 μM B[a]P, respectively (Fig. 8A, B). In this assay, *Salmonella typhimurium* strain TA100 was also used, but we did not find a significant difference between the two strains (Data are not shown).

Addition of α-naphthoflavone, a chemical inhibitor of the CYP1A subfamily, significantly inhibited this mutagenic activity in a concentration-dependent manner. This inhibition was especially noticeable at 20 μM, when the inhibition percentages were 50%, 51%, 57%, and 52% in rats deer, cattle, and horses, respectively (Fig. 9A). In a trial to inhibit the mutagenic activity by immunoinhibition, after incubation for 30 min with the microsomes, all animals examined showed cross reactivity with the anti-rat CYP1A1 antibody used in this study. Anti-rat CYP1A1 antibody inhibited the B[*a*]P mutagenic activation in all animals in a concentration-dependent manner. Significant inhibition was clear when the concentration of the antibody was 10 μg because the inhibition percentages were 83%, 80%, 70%, and 83% in rats, deer, cattle and horses, respectively (Fig. 9B).

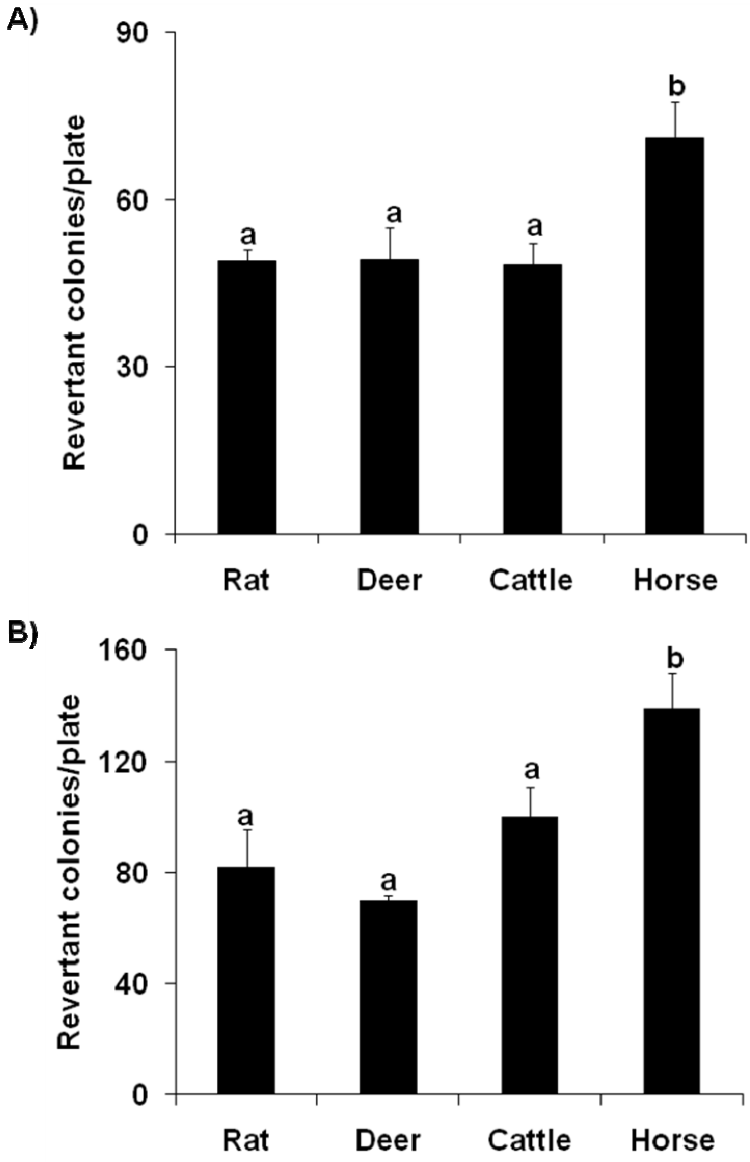


Fig. 8. Mutagenic activation activity of CYP1A in the meat producing-animals and rats

The number of revertant colonies reflects the mutagenic activation activity of CYP1A in the microsomes of different meat-producing animals compared to rats when benzo[*a*]pyrene is used as the substrate with (A) 1μM or (B) 10μM. The data represent the means ± SD for five animals from each species. Identical letters are not significantly different from each other. $P<0.05$.

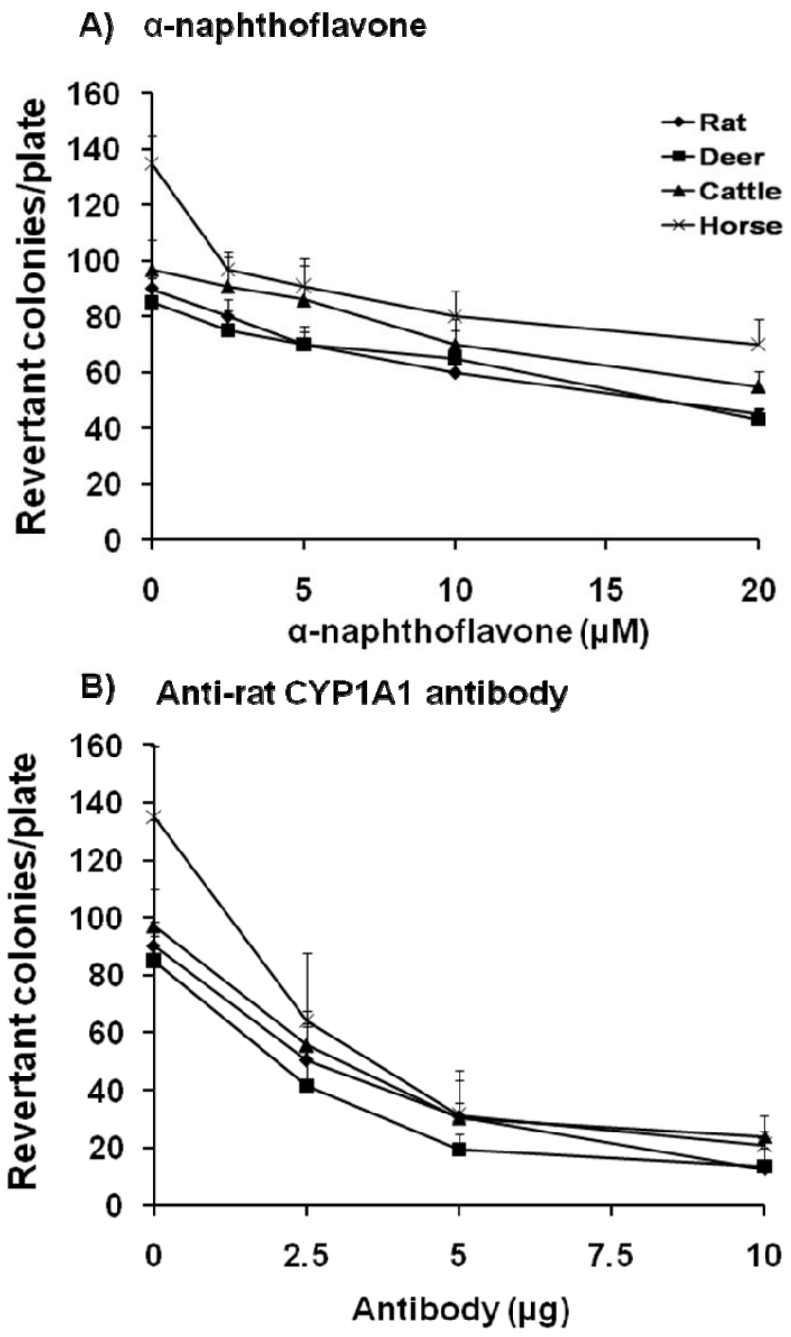


Fig. 9. Inhibition of B[*a*]P mutagenic activation in three meat-producing animals and rats by CYP1A specific inhibitors

A) Effect of α-naphthoflavone on B[*a*]P (10μM) mutagenic activation in three meat-producing animals compared to rats, under different concentrations of the inhibitor, α-naphthoflavone, (2.5, 5, 10, and 20 μM). The data represent the means ± SD for five animals from each species. B) Effect of anti-rat CYP1A1 antibody on B[*a*]P (10μM) mutagenic activation in cattle, deer and horses hepatic microsomes compared to rats, under different concentrations of the inhibitor anti-rat CYP1A1 antibody (2.5, 5, and 10 μg) after incubation of both microsomes and the antibody for 30 min at room temperature. Data at 0 concentration represent the B[*a*]P (10μM) mutagenic activation without addition of antibody. Rabbit IgG was used as a negative control with the following concentrations (2.5, 5, and 10 μg) instead of anti-rat CYP1A1 antibody in all animals (data are not shown in this figure). The data represent means ± SD of three experiments performed at different times using liver microsomes of five animals of each species. The data at concentrations of 2.5, 5, and 10 μg of the antibody are significantly different from the data at concentration of 0 μg antibody in all animals. $P<0.05$.

To investigate the contribution of other cytochrome P450 subfamilies in this activity, the liver microsomes were incubated with anti-rat CYP3A2 and anti-rat CYP2E1 antibodies for 30 min prior to the reaction; however, we did not find any significant inhibition in all species examined, as shown in Figures 10A and 10B, suggesting that these subfamilies have no role in this metabolic activity under these conditions. The inhibitors did not show any significant effect on the cell viability in the absence of the liver microsomes.

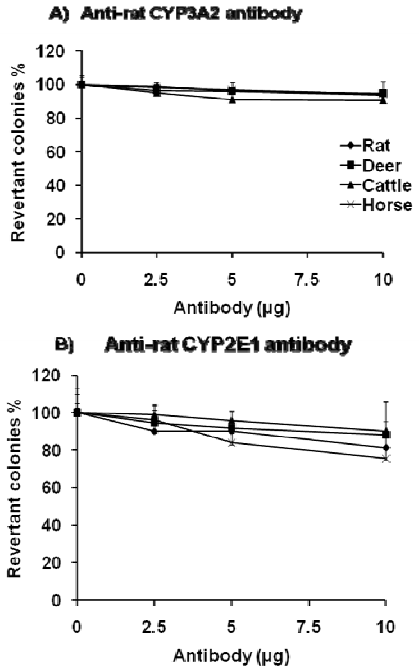


Fig. 10. Immunoinhibition of B[*a*]P mutagenic activation in three meat-producing animals and rats by inhibitors of other CYPs

A) Effect of anti-rat CYP3A2 antibody on B[*a*]P (10µM) mutagenic activation in cattle, deer and horse hepatic microsomes compared to rats under different concentrations (2.5, 5, and 10 µg) after incubation of both microsomes and the antibody for 30 min at room temperature. Data at 0 concentration represent the B[*a*]P (10µM) mutagenic activation without addition of antibody. Rabbit IgG was used as a negative control with the following concentrations (2.5, 5, and 10 µg) instead of anti-rat CYP3A2 antibody in all animals (data are not shown in this figure). The data represent means ± SD of three experiments performed at different times using liver microsomes of five animals. B) Effect of anti-rat CYP2E1 antibody on B[*a*]P (10µM) mutagenic activation in cattle, deer and horse hepatic microsomes compared to rats under different concentrations (2.5, 5, and 10 µg) after incubation of both microsomes and the antibody for 30 min at room temperature. Data at 0 concentration represent the B[*a*]P (10µM) mutagenic activation without addition of antibody. Rabbit IgG was used as a negative control with the following concentrations (2.5, 5, and 10 µg) instead of anti-rat CYP2E1 antibody in all animals (data are not shown in this figure). Data represent means ± SD of three experiments performed at different times using liver microsomes of five animals. $P<0.05$.

Role of phase II enzymes against B[a]P mutagenic activation in meat producing animals and rats

The addition of cofactors of phase II enzymes such as UGA, a co-factor of UGT, significantly reduced the production of revertant colonies in a concentration-dependent fashion. At the highest concentration, 10 mM, the production of the revertants was reduced to 63%, 64%, 55%, and 42% in rats, deer, cattle and horses, respectively, as shown in Figure 11A. The addition of GSH, a co-factor for GST, also significantly reduced the production of the revertants in a concentration-dependent manner. At 30 mM GSH, the production of the colonies was reduced to 65%, 58%, 76%, and 49% in rats, deer, cattle and horse S9 fractions, respectively (Fig. 11B). The co-factors did not show any significant effect on cell viability in the absence of the liver microsomal or S9 fractions.

To determine the other phase II enzymes activities, specific substrates were used. In the case of UGT, ρ-nitrophenol was used as a specific substrate, but no significant difference was found in the UGT activity in the liver microsomes of cattle, deer, and horses compared to rats because their activities were 14.0 ± 2.0, 11.3 ± 1.1, 12.7 ± 3.1, and 14.7 ± 4.2 nmol/min/mg microsomal protein, respectively (Fig. 12A). CDNB was used as the specific substrate to measure the cytosolic GST activity in these animals; horses had the highest activity at 1.2 ± 0.1 after rats at 1.5 ± 0.3 compared to cattle and deer, whose GST activities were 0.6 ± 0.1 and 0.6 ± 0.1 μmol/min/mg cytosolic protein, respectively (Fig. 12B).

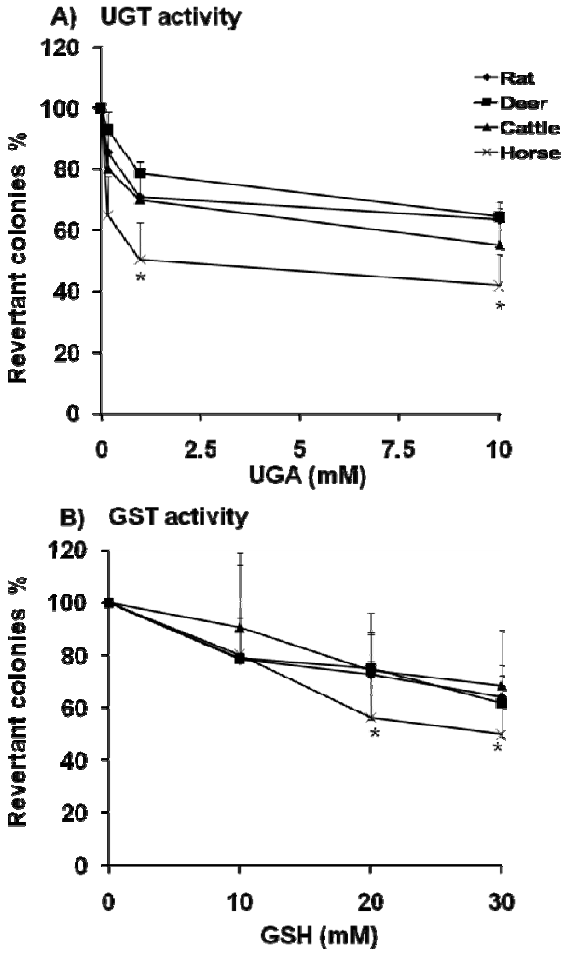


Fig. 11. Effect of UGT and GST on B[*a*]P mutagenic activation in three meat-producing animals and rats

A) Effect of UGT on B[*a*]P (10μM) mutagenic activation in cattle, deer and horse hepatic microsomes compared to rats under different concentrations of UGA, a co-factor of UGT (0.2, 1, and 10 mM). Data at 0 concentration represent the B[*a*]P (10μM) mutagenic activation without adding UGA. Data represent means ± SD of three experiments performed at different times using liver microsomes of five animals of each species. B) Effect of GST on B[*a*]P (10μM) mutagenic activation in cattle, deer and horse hepatic S9 compared to rats under different concentrations (10, 20, and 30 mM) of GSH, a co-factor of GST. Data at 0 concentration represent the B[*a*]P (10μM) mutagenic activation without addition of GSH. Data represent means ± SD of three experiments performed at different times using liver S9 of five animals of each species. $P<0.05$.

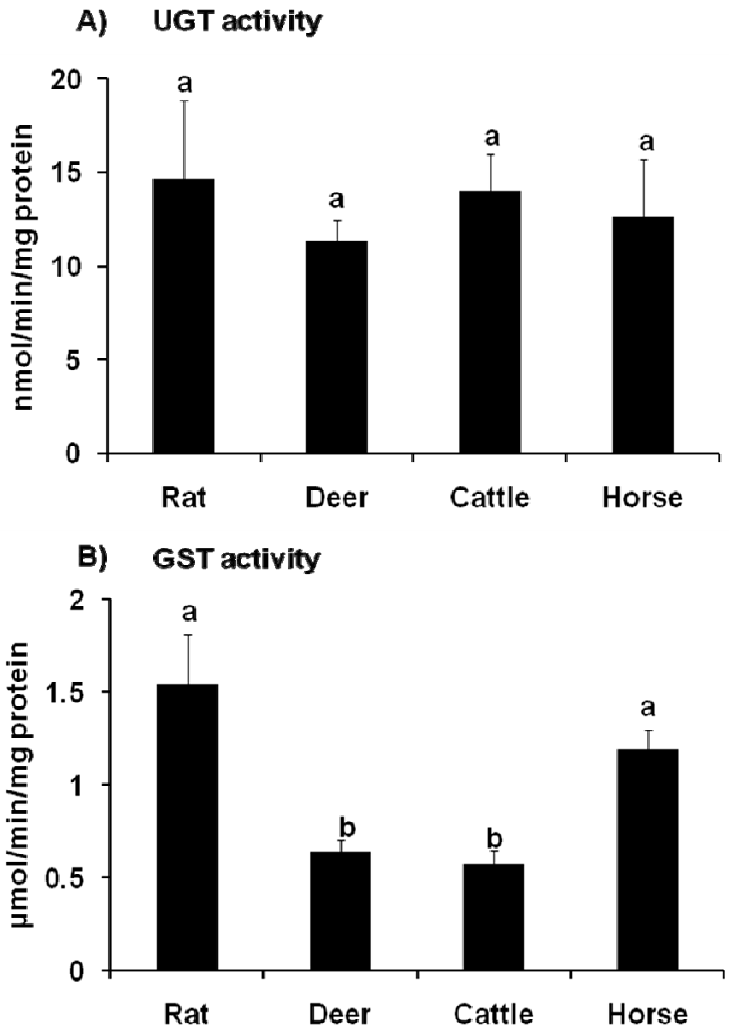


Fig. 12. UGT and GST activities in three meat-producing animals and rats

A) UGT activity towards ρ-nitrophenol (nmol/min/mg protein) of hepatic microsomes from cattle, deer and horses compared to rats, as determined by the assay described in the Methods section. Data represent means ± SD of three experiments performed at different times using liver microsomes of five animals of each species. Identical letters are not significantly different from each other. $P<0.05$. B) GST activity towards 1-chloro-2,4-dinitrobenzene (µmol/min/mg protein) of hepatic cytosols from cattle, deer and horses compared to rats, as determined by the assay described in the Methods section. Data represent means ± SD of three experiments performed at different times using cytosol of five animals of each species. Identical letters are not significantly different from each other. $P<0.05$.

Discussion

Cytochrome P450, a superfamily of heme proteins, is involved in the metabolism of a vast array of carcinogens, drugs and endogenous compounds. The liver is the major organ involved in P450-mediated xenobiotic metabolism (Conney, 2003). CYP1A1, a member of the P450 superfamily, contributes notably to the toxicity of many carcinogens, especially polycyclic aromatic hydrocarbons, because it is the principal enzyme that bioactivates inert hydrocarbons into DNA-binding reactive metabolites (Kommaddi et al., 2007). Polycyclic aromatic hydrocarbons are ubiquitous environmental carcinogens that have been shown to become carcinogenic only after metabolic activation by cytochrome P450, especially CYP1A1 (Conney, 1982; Shimada, 2006).

Cattle, deer and horses are important sources of animal-derived food products; nonetheless, our understanding about the expression of drug-metabolizing enzymes, either phase I or II enzymes, in food-producing animals still remains superficial, despite the obvious toxicological consequences (Giantin et al., 2008). Although several separate studies have investigated the drug metabolizing enzymes in deer, cattle, or horses as shown by several researchers (Machala et al., 2003; Nebbia et al., 2003; Sivapathasundaram et al., 2001; Sivapathasundaram et al., 2003a; Darwish et al., 2010a), no study of the ability of the liver microsomes of these animals to mutagenically activate promutagens has been reported. Tometsko et al. (1981) found that chickens had the highest B[*a*]P activation level compared to rats, followed by cattle, pigs and sheep. Smith et al. (2007) quantitatively compared the different testicular and hepatic microsomal metabolisms of B[*a*]P in rats, mice, hamsters, rams, boars, bulls, and monkeys. Also, Harris et al. (2009) reported the concentrations of B[*a*]P different

metabolites generated by the microsomes of liver and ovaries of rats, mice, goats, sheep, pigs, and cows. Higher CYP1A-dependent activity and protein expression were observed in these animals than in rats (Machala et al., 2003; Nebbia et al., 2003; Sivapathasundaram et al., 2001; Sivapathasundaram et al., 2003a; Darwish et al., 2010a), and higher epoxide hydrolase activity, a very important enzyme in the B[*a*]P metabolic pathway, was observed in ungulates compared to rats (Sivapathasundaram et al., 2003b). These activities may indicate a high risk of mutagenesis and carcinogenesis in these ungulates if they are exposed to promutagens. Thus, we performed this study not only to investigate the ability of cattle, deer and horses to activate B[*a*]P, an ideal promutagen, but also to investigate the contribution of phase II enzymes against this mutagenic activity and the mechanism of protection against the adverse effects produced by phase I metabolism. In addition, other phase II enzymes activities in this group of animals were studied in comparison to rats.

In this chapter, I found that the liver microsomes of these animals mutagenically activated B[*a*]P in the Ames mutagenicity assay. Interestingly, horse liver microsomes had the highest ability to produce revertant colonies compared to the other animals examined. Consistent with this finding, Nebbia et al. (2003) and Darwish et al. (2010a) found that horse liver microsomes had the highest EROD, CYP1A-dependent activity compared to several agricultural and laboratory animals. This correspondence may indicate that the high mutagenic activation of B[*a*]P in horses is attributable to the high CYP1A activity in this animal species. To confirm that this mutagenic activity was because of the CYP1A subfamily, I studied the inhibition of this activity by chemical means, using a specific inhibitor of the CYP1 family, α-naphthoflavone, or by immunological means, using the anti-rat CYP1A1 antibody. These inhibitors inhibited this

mutagenic activity in a concentration-dependent manner. In order to evaluate the contribution of other cytochrome P450 subfamilies to this activity, I used anti-rat CYP3A2 and anti-rat CYP2E1; however, these antibodies had no significant effect on the mutagenic activity. This result highly suggests that as in the rat, the mutagenic activity of B[*a*]P in the meat-producing animals examined is mainly because of the CYP1A subfamily. In correspondence with my finding, Shimada and Fuji-Kuriyama (2004) declared that CYP3A4, 2A6, 2C9, and 2C19 had no significant contribution on the mutagenic activation of B[a]P in mice using umu gene expression test. While CYP1A1 metabolism generates reactive metabolites, the importance of CYP1B1, which is mostly extrahepatic, cannot be ruled out. As, Kim et al. (1998) indicated that CYP 1B1 carries out metabolism of B[*a*]P along the pathway to the postulated ultimate carcinogen, the diol epoxide 2, at rates much higher than P450 1A2 but less than P450 1A1 in rodent tumor models and in humans.

In rats and humans, phase II enzymes like UGT and GST play an important role in protection from the adverse effects produced by the metabolites of phase I, as reported previously by Fujita et al. (1988) and Shimada, (2006). To affirm the effects of the phase II enzymes against this mutagenic activity in the meat-producing animals, I added GSH and UGA, co-factors for GST and UGT, to the reaction mixture in the Ames assay. Phase II enzymes reduced the production of the revertant colonies in a concentration-dependent fashion in all examined animals, and this result agrees with that obtained by Fujita et al. (1988) in rat experiments. Surprisingly, the highest significant reduction percentage was recorded in horses, suggesting that horses had also the highest UGT- and GST- dependent activities of all the examined animals. This finding may explain in part how these animals, and in particular horses,

protect themselves from the adverse effects of environmental pollutants. Moreover, Shimada et al. (2007) reported that the parent polycyclic aromatic hydrocarbons may act as inhibitors of further metabolism of their own metabolites to prevent the carcinogenic effects either through direct inhibition or in a competitive mechanism.

Because of the lack of information about the activities of phase II enzymes in these meat-producing animals, and to confirm the high UGT- and GST- dependent activities in horses compared to cattle, deer and rats, I investigated the UGT and GST activities using their specific substrates. In the case of UGT, I used ρ-nitrophenol as a specific substrate, but I did not find any significant differences between horses and the other animals examined compared to the results of the Ames assay. CDNB was used as a specific substrate for GST activity. Horses also had the highest GST activity compared to other meat-producing animals.

In conclusion, although environmental pollution endangers meat-producing animals in many locations around the world, and although this may lead to mutagenesis and/or carcinogenesis, the bodies of these animals adapt to these changes by increasing the activities of phase II enzymes. Phase II enzymes, particularly in horses, play a vital role in producing a state of balance between the bioactivation and bioinactivation of environmental chemicals in living organisms.

Chapter IV

Carotenoids as regulators for inter-species difference in Cytochrome P450 1A expression and activity in the meat-producing animals

Introduction

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, 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 μM (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., 2003a; Darwish et al., 2010a). 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.

In this chapter, I investigate the role of the accumulated carotenoids and retinoids in the inter-species differences in CYP1A-dependent activity and protein expression among deer, cattle, 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.

Materials and methods

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 goat 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 [B*a*A], Chrysene [Chr], Benzo[*e*]pyrene [B*e*P], Benzo[*b*]fluoranthene [B*b*F], Benzo[*k*]fluoranthene [B*k*F], Benzo[*a*]pyrene [B*a*P],Dibenz[*a,h*]anthracene [D*ah*A], Benzo[*ghi*]perylene [B*ghi*P]) 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).

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 sika deer (*Cervus nippon yesoensis*) were hunted from wildlife (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, soya bean, 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.

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 37ºC in a humified incubator with 5 % CO_2 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 sulpfoxide (DMSO) was used as a negative control.

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.

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.

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 $MgCl_2$, 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.

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 and H_2O_2 (0.025% 3,3-diaminobenzidine, 0.0075% H_2O_2Tris-HCl buffer, pH 7.4). Intensities of the immunoreactive bands were densitometrically analyzed using the public domain NIH Image program (National Institutes of Health, MD, USA).

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 re-dissolved in ethanol and the absorption measured spectrophotometrically. A value of 2620 was used for the extinction coefficient ($E^{1\%}$) at 450 nm.

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 yeilds 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 N_2 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. Five microliter 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.

RNA extraction

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

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 70ºC 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 30ºC for 10 min, 42ºC for 1h and 90ºC for 10 min to prepare the cDNA.

Quantitative real-time RT-PCR (qPCR) 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 GCTCAAGTACCCCCATGTCG and antisense ATCAGTGTATGGCATTTTACTGCGG (accession number NP-001128316) and the β-actin sense ATGTACGTAGCCATCCAGGC and antisense TCCACACAGAGTACTTGCGC (accession number V01217). The reaction mixture (final volume 20 μL) for the PCR was prepared with 1x Master Mix reagents, 300 nM of each primer, 500 ng cDNA, 1x ROX reference dye in 1 μL 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 95ºC for 15 min, denaturation at 95ºC, annealing at 60ºC for 1 min for CYP1A1 and at 62ºC for 1 min for CYP2B1, and at 72ºC 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.

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 55ºC. 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 [B*a*A], Chrysene [Chr], Benzo[*e*]pyrene [B*e*P], Benzo[*b*]fluoranthene [B*b*F], Benzo[*k*]fluoranthene [B*k*F], Benzo[*a*]pyrene [B*a*P], Dibenz[*a,h*]anthracene [D*ah*A], Benzo[*ghi*]perylene [B*ghi*P]) were determined from calibration curves made by the standards (Accus Standard Inc.).

Statistical analysis

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

Results

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. 13A).

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 (Fig. 13B).

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, cattle, deer and rats was 6.8 ± 0.6, 7.6 ± 0.5, 3.7 ± 0.2 and 0.9 ± 0.2 µg/g, respectively (Fig. 14A). 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. 14B, C).

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 µg/g) and horses (6.4 ± 0.6 µg/g) had the highest content of β-carotene followed by deer (3.6 ± 0.2 µg/g). No β-carotene was detected in the livers of the examined rats (Fig. 15A). Scatter plots between β-carotene content and EROD activity as well as the protein expression level of CYP1A1 showed positive correlations (Fig. 15B, C).

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 ± 19 µg/g) followed by cattle (151.2 ± 15.9 µg/g), horses (61.6 ± 6.3 µg/g) and deer (58.3 ± 13.7 µg/g) (Fig. 16A). Scatter plots between the retinol content and both EROD activity and protein expression levels of CYP1A1 showed negative correlations (Fig. 16B, C).

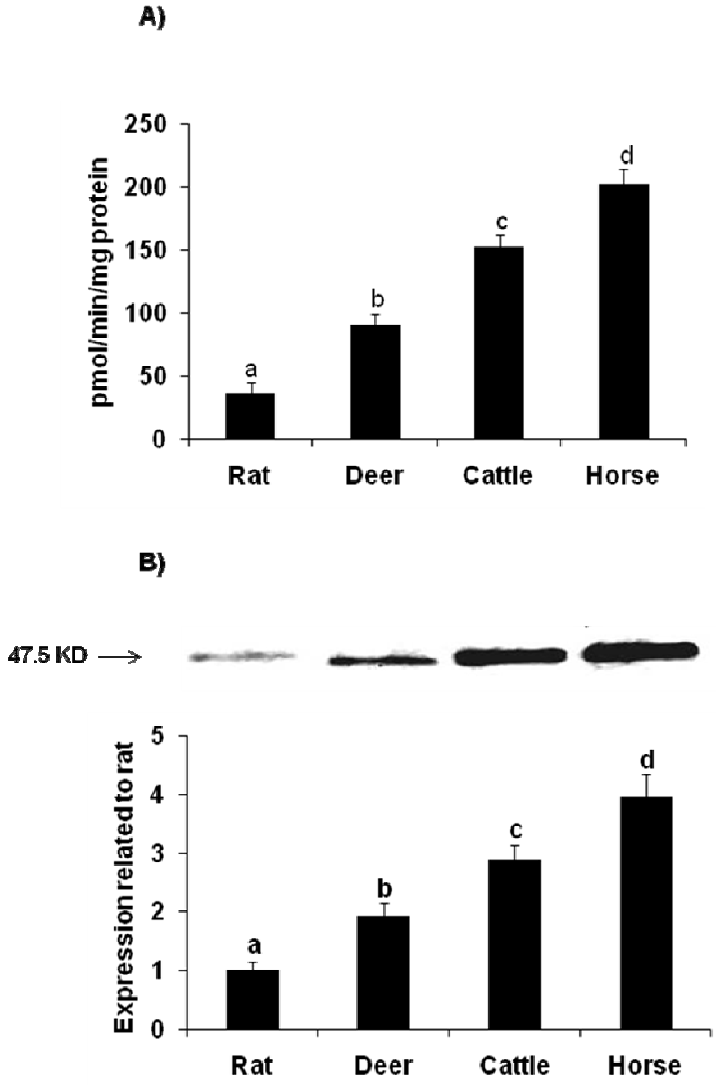


Fig. 13. CYP1A dependent EROD activity and protein expression in ungulates and rats

A) EROD activity (pmol/min/mg protein) in different ungulates compared to rats using a substrate concentration of 2.5 μM. The data represent the means ± 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 (15μg/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.

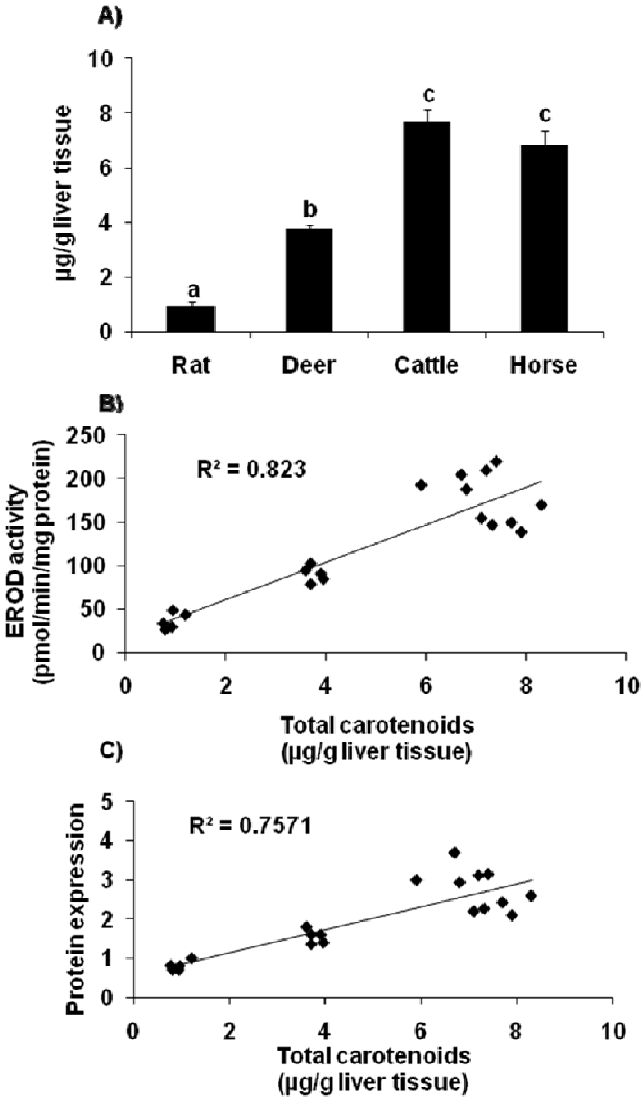


Fig. 14. Relationship between total carotenoid content and CYP1A

A) Total carotenoid content in the liver of several ungulates as well as in rats. The total carotenoid content (µg/g) was measured spectrophotometrically. A value of 2620 was used as the extinction coefficient ($E^{1\%}$) at 450 nm. The data represent the means ± 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 (µg/g liver tissue) and CYP1A protein expression level in the examined ungulates and in rats.

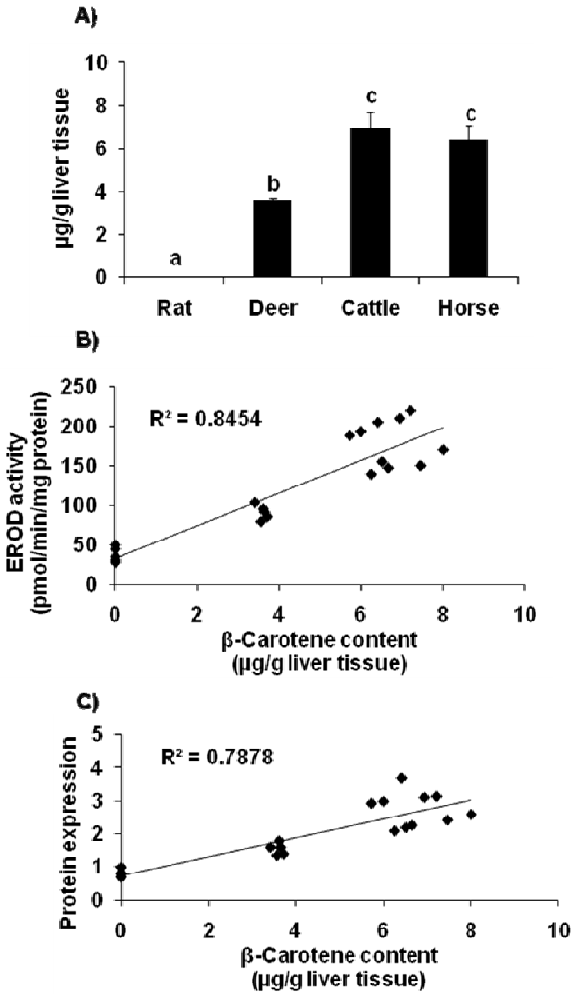


Figure 15. Relationship between β-carotene content and CYP1A

A) β-carotene content (µg/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 mm × 2.1 mm φ, (5µm). 5 µl 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 means ± 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 (µg/g liver tissue) and EROD activity (pmol/min/mg protein) in the examined ungulates and in rats. C) Scatter plots between β-carotene content (µg/g liver tissue) and CYP1A protein expression levels in the examined ungulates and in rats.

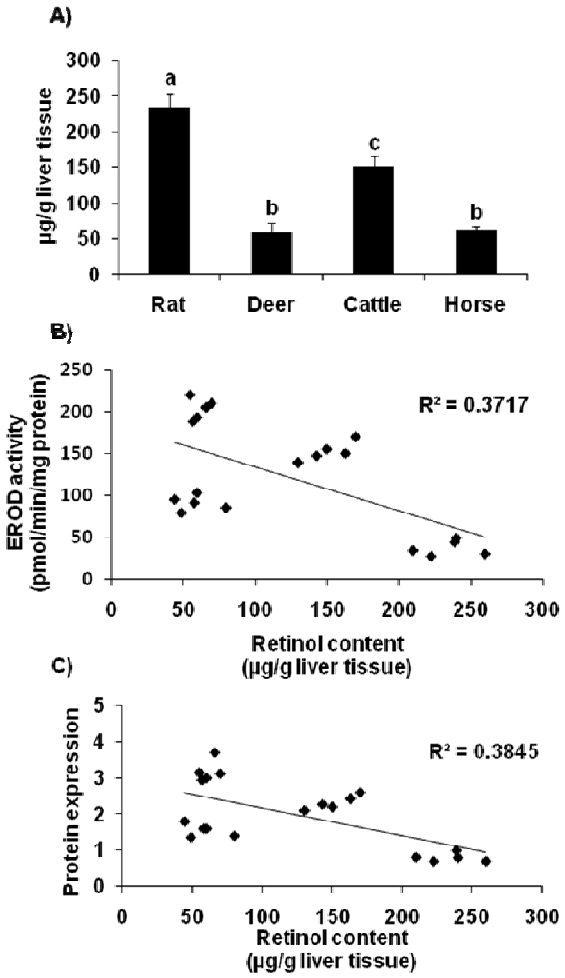


Fig. 16. Relationship between retinol content and CYP1A

A) Retinol content (µg/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 mm × 2.0 mm φ, (5 µm). 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) 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 means ± 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 (µg/g liver tissue) and EROD activity (pmol/min/mg protein) in ungulates and in rats. C) Scatter plots between retinol content (µg/g liver tissue) and CYP1A protein expression levels in the examined ungulates and in rats.

CYP1A1and CYP2B1 mRNA expressions 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 rats. 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. 17A). 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. 17B). In contrast, neither rat cells treated with different carotenoid extracts, nor those treated with β-carotene and retinol showed any significant difference with the untreated ones in CYP2B1 expression (Data are not shown).

CYP1A1protein 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 rats. 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. 18A). 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. 18B).

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 3).

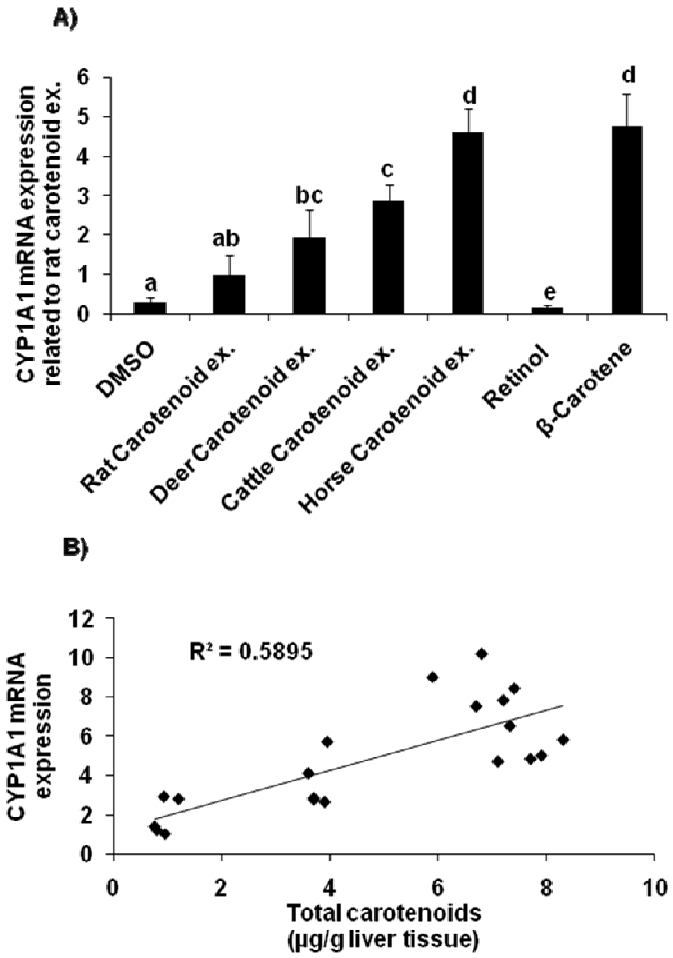


Fig. 17. CYP1A1 mRNA expression in H4-II-E rat cells and its correlation with total carotenoid content in the liver of various examined ungulates as well as rats.

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 means ± SD. Identical letters were not significantly different from each other. $P < 0.05$. B) Scatter plots between total carotenoid content (µg/g liver tissue) and CYP1A1 mRNA expression in the treated H4-II-E rat cells with different carotenoid extracts.

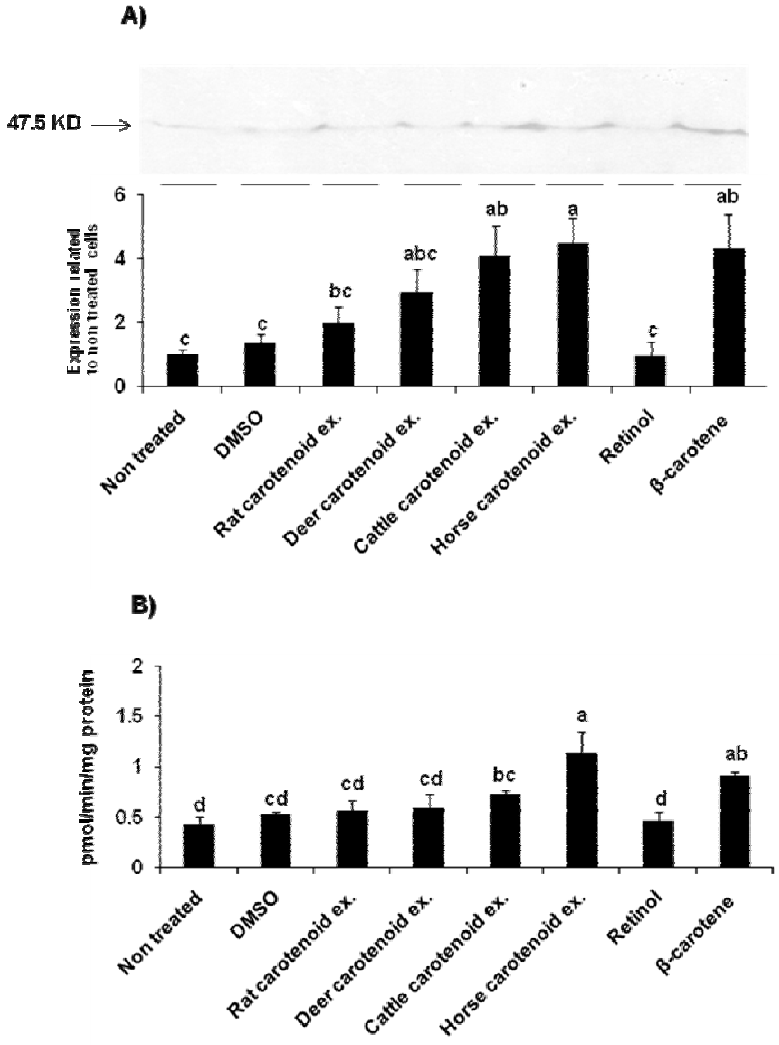


Fig. 18. CYP1A protein expression and dependent EROD activity in H4-II-E rat cells

A) CYP1A protein expression in H4-II-E rat cells treated with different carotenoid extracts. Whole cell lysate protein samples (15µg/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 (pmol/min/mg protein) in H4-II-E rat cells treated with different carotenoid extracts.using a substrate concentration of 2.5 µM. The data represent the means ± SD for five treatments from each extract. Identical letters were not significantly different from each other. P <0.05.

Table 3: 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
B*a*A	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
B*e*P	0.14±0.01	0.23±0.24	0.09±0.03	0.06±0.03
B*b*F	0.18±0.01	0.24±0.12	0.06±0.08	0.02±0.00
B*k*F	0.55±0.02	0.55±0.15	0.13±0.00	0.33±0.13
B*a*P	0.01±0.004	0.09±0.01	0.01±0.00	0.02±0.01
D*ah*A	0.38±0.11	0.09±0.08	0.01±0.00	0.01±0.01
B*ghi*P	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.

Discussion

In this chapter, I defined the metabolic activity and protein expression of CYP1A in meat-producing animals such as cattle, deer and horses, compared with the rat as the control. EROD activity is routinely used to monitor CYP1A activity (Ioannides, 1999; Sivapathasundaram et al., 2003a). In the equine liver, ethoxyresorufin O-deethylase showed the highest activity when compared with the rats, 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 with 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 the second chapter, EROD kinetic parameters were investigated (Darwish et al. 2010a), but I found very small differences in the catalytic efficiency between the animals examined. As the Vmax/Km 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 3, 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 rats. 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., 1996 a,b). In addition, some carotenoids such as astaxanthin, canthaxanthin, β-carotene and bixin are considered as inducers for CYP1A (Jewell and O'Brien, 1999). Especially, I 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, I 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 I 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 my 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 into retinol in the case of rats. Especially, that rat diet contained wheat, corn, soya bean 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 finding, rat cells were exposed to different carotenoid extracts, in addition to β-carotene and retinol, which were used as control chemicals. I 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, I suggest that 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 (Fig. 19). Further approaches are needed to investigate the transcriptional mechanisms behind either induction or reduction of CYP1A by the accumulated carotenoids and retinoids.

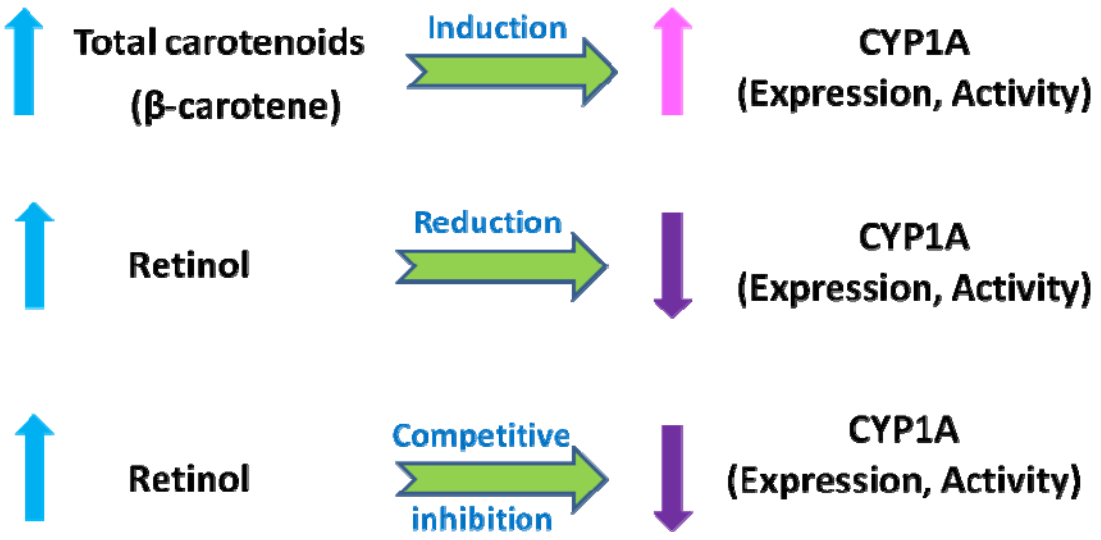


Fig. 19. Summary of the regulatory effects of carotenoids and retinoids on CYP1A expression and activity in ungulates and rats

Chapter V

Expression and sequence of CYP1A1 in the camel

Introduction

Egypt, with an estimated population of 80 million, is one of the most populous countries in Africa. Environmental pollution is considered to be one of the most dangerous hazards affecting not only Egypt but also the majority of world countries. By the beginning of 1950s, heavy industries were born in Egypt along the Nile Delta in Cairo and other metropolitan areas. As a result environmental pollution with polycyclic aromatic hydrocarbons, dioxins and dioxin-like compounds has increased due to increase in population, agricultural projects, industrial and other activities along the Nile Delta. To monitor the impact of such pollution on the public health, we have to establish some biomarkers for this pollution. Meat-producing animals are considered to be good biomarkers for different environmental pollutants, simply because they have the ability to accumulate some residues of either the pollutants or their metabolites (Hyyti et al., 2001).

The camel has become domesticated and is classified as a meat- and milk-producing animal in many parts of the world, while in other places, this species is still wild. In Egypt, camel had been domesticated for long time and used as a good source for meat, milk and hide production. As I mentioned before, induction of CYP1A subfamily is considered as a good

bioindicator for the pre-slaughter exposure to different xenobiotics. The use of CYP1A induction as an assessment technique has increased in recent years. This is due mainly to the optimization of protocols for rapid and relatively inexpensive measurement of its expression and activity (Burke et al., 1994). Nevertheless, there is a lack of information about CYP1A, particularly molecular information, in this valuable animal.

Like other mammals, the camel exhibits CYP-dependent activities in the liver and extrahepatic tissues (Raza and Montague, 1993; Raza and Montague, 1994; Raza et al., 1998; Elsheikh et al., 1999).

In this chapter, I identified a partial sequence and the tissue distribution of CYP1A1 in different tissues of the camel. This study also may reflect the biological response of camel towards variant xenobiotics.

Materials and methods

Chemicals and reagents

All test reagents used were of reagent grade including those described below. TRI reagent was purchased from Sigma (St Louis, MO, USA). Oligo(dT) primer, RT-buffer and ReverTra Ace were purchased from TOYOBO (Osaka, Japan). Primer sets were purchased from Invitrogen (Carlsbad, CA, USA). Ex Taq Polymerase was purchased from TaKaRa (Tokyo, Japan) All other reagents were analytical grade or the highest quality available and purchased from Wako Pure Chemical Industries, Tokyo, Japan.

Animals

All experiments using animals were performed according to the guidelines of the Hokkaido University Institutional Animal Care and Use Committee. Camel tissue samples (liver, heart, spleen, kidney, prescapular lymph node, lung, hump, and tongue) from at least three 3- to 5-year-old male Arabian camels (*Camelus dromedarius*) were purchased from Egypt with permission from the Ministries of Agriculture and animal quarantine departments of both Egypt and Japan.

RNA extraction

Total RNA was prepared from each tissue by the single-step method (Chomczynski and Sacchi, 1987), using TRI reagent. from Sigma Chemical Co. (St. Louis, MO, USA). The

concentration and purity of the RNA fraction was determined spectrophotometrically at 260 and 280 nm, respectively.

Oligonucleotides

Degenerate primers (Invitrogen, Carlsbad, CA, USA) were designed based on the conserved regions of eight mammalian CYP1A sequences retrieved from GenBank (Table 4). The sense primer (5′-TCT TTG GRG CWG GNT TTG ACA C -3′) and the anti-sense primer (5′- TGG TTR AYC TGC CAC TGG TT-3′) were both positioned upstream from the well-described cysteine-containing heme-binding region (Teramitsu et al., 2000).

Table 4. Conserved regions used to design degenerate primers

Gene	Accession number	Primer regions (bp)	
		Upstream	Downstream
Mouse CYP1A1	(K02588)	953-974	1240-1259
Guinea pig CYP1A1	(D11043)	929-950	1216-1235
Monkey CYP1A1	(D17575)	941-962	1228-1247
Human CYP1A1	(K03191)	941-962	1228-1247
Mouse CYP1A2	(K02589)	935-956	1222-1241
Guinea pig CYP1A2	(D50457)	941-962	1228-1247
Hamster CYP1A2	(M34446)	935-956	1222-1241
Human CYP1A2	(M38504)	941-962	1228-1247

cDNA amplification and isolation

Oligo(dT) primed total RNA was reverse-transcribed to cDNA using ReverTra Ace (TOYOBO Co. Ltd., Osaka, Japan). The resultant cDNA was amplified with the aforementioned degenerate primers and Ex Taq Polymerase (TaKaRa, Tokyo, Japan) by PCR on a TaKaRa PCR Thermal Cycler PERSONAL (TaKaRa, Tokyo). The thermal cycle of the reaction was started with a single 4 min cycle at 94°C, followed by 40 cycles of 15 sec denaturation at 94°C, 45 sec annealing at 54°C, and 1 min extension at 72°C. A single major band of 300 bp was detected by analytical electrophoresis on a 1.5% agarose/ethidium bromide (0.25 μg/ml) gel. The PCR products were cloned into the pCR 2.1 vector (Invitrogen), followed by transformation of *E. coli* DH5-α with the plasmid DNA. The bacterial cells were cultured for 14 h average. Blue-white screening was employed to identify transformed cells, and the cDNA inserts of 300 bp were amplified by direct PCR using M13 primers (Invitrogen), specifically targeting the multiple cloning site of the vector. The cells from the selected colonies were cultured for another 9 h in liquid Luria-Bertani broth. The plasmids were purified by a plasmid miniprep method using a GenElute Mikniprep Kit (Sigma, St Louis, MO, USA).

DNA sequencing

Purified plasmids were directly sequenced by the dye terminator cycle sequencing method. The cycle sequencing was performed on a Program Temp Control System PC-700 (ASTEC) with the vector-specific M13 Primers, RV-N and M3 (TaKaRa) using the ABI PRISM Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster, CA). The nucleotide sequences were determined by an automated DNA Sequencer (ABI PRISM 310

genetic analyzer). A minimum of four clones were sequenced in both forward and reverse directions to avoid sequence ambiguities.

Phylogenetic analysis of mammalian CYP1A genes

The cDNA and amino acid sequences of camel CYP1A1 were aligned with those of other mammals using CLUSTALW 1.83 program. Phylogenetic trees for amino acid sequences were constructed by the neighbor-joining (NJ) method using the PHYLIP program.

Tissue distribution of CYP1A1 in different tissues of camel

To identify the tissue distribution of CYP1A1 in different tissues of the camel, the resultant cDNA was amplified with gene specific sense (5′-TTT GGA GCT GGG TTT GAC ACA-3′) and anti-sense (5′-TTG ATC TGC CAC TGG TTC ACA-3′) primers. The thermal cycle of the reaction was started with a single 1 min cycle at 94°C, followed by 40 cycles of 10 sec denaturation at 94°C, 30 sec annealing at 59°C and 1 min extension at 72°C. A single band of 198 bp was detected by analytical electrophoresis on a 1.5% agarose/ethidium bromide (0.25 µg/ml) gel. The cDNA was also amplified with the specific primers of camel glyceraldehyde-3-phosphate dehydrogenase (G3PDH, accession number EU331417) as a housekeeping gene; the sense primer was set as 5′-AAG AAG GTA GTG AAG CAG GCA-3′ and the anti-sense primer (5′-TTT TTT TCT CGG GTT GAG TG-3′). The thermal cycle of the reaction was started with a single 1 min cycle at 94°C, followed by 25 cycles of 10 sec denaturation at 94°C, 1 min annealing at 60°C and 1 min extension at 72°C. A single band of 444 bp was detected by electrophoresis. The intensity of CYP1A1 was compared to those of the liver with correction by

G3PDH expression using the public domain NIH Image program (National Institutes of Health, Bethesda, MD, USA). The tissue distribution of the mRNA expression of CYP1A1 was expressed as the mean ± standard deviation (SD) of three animals.

Statistical analysis

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

Results

Sequence and phylogenetic analysis of CYP1A1 in camel

I succeeded in amplifying CYP1A1 from a cDNA fragment from the camel liver and obtained a partial sequence of camel CYP1A1, as indicated in fig. 20. By studying the homology of the isolated camel CYP1A1 with CYP1A isoforms of other ungulates, I found that camel CYP1A1 showed the highest similarity with sheep and cattle CYP1A1 (94%), followed by horse CYP1A1 (87%) and pig CYP1A1 (86%), as shown in Table 5.

In the phylogenetic analysis of camel CYP1A1, my isolated clone located beside sheep and cattle CYP1A1 as indicated in fig. 21.

Table 5. Homology of the camel CYP1A1 deduced amino acid sequence with other ungulate CYP1A genes

Animal species	Gene	Accession number	Homology %
Cattle	CYP1A1	XP-588298	94%
Sheep	CYP1A1	P56591	94%
Horse	CYP1A1	XP-001493959	87%
Pig	CYP1A1	XP-001927881	86%
Rat	CYP1A1	NP-036672	87%
Cattle	CYP1A2	NP-001092834	78%
Horse	CYP1A2	XP-001493936	80%
Pig	CYP1A2	XP-001927857	80%

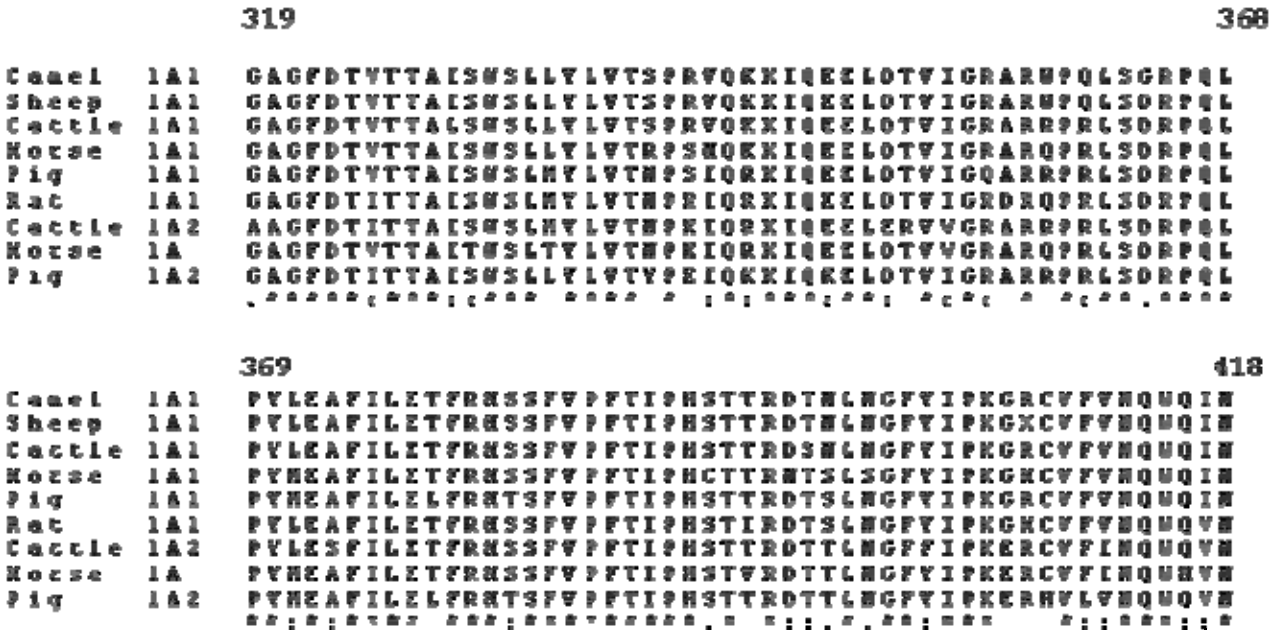


Fig. 20. Alignments of deduced amino acid sequences of CYP1A1 of the camel, ungulates and the rat

Asterisks indicate the regions conserved among all seven sequences in the alignment. The colons indicate that strong groups are fully conserved, and periods indicate they are “weakly conserved”. The predicted amino acid sequences are shown aligned with the rat CYP1A1 sequence of the corresponding region. The sequence obtained corresponds to the heme-binding site from substrate recognition sites in the gene construction.

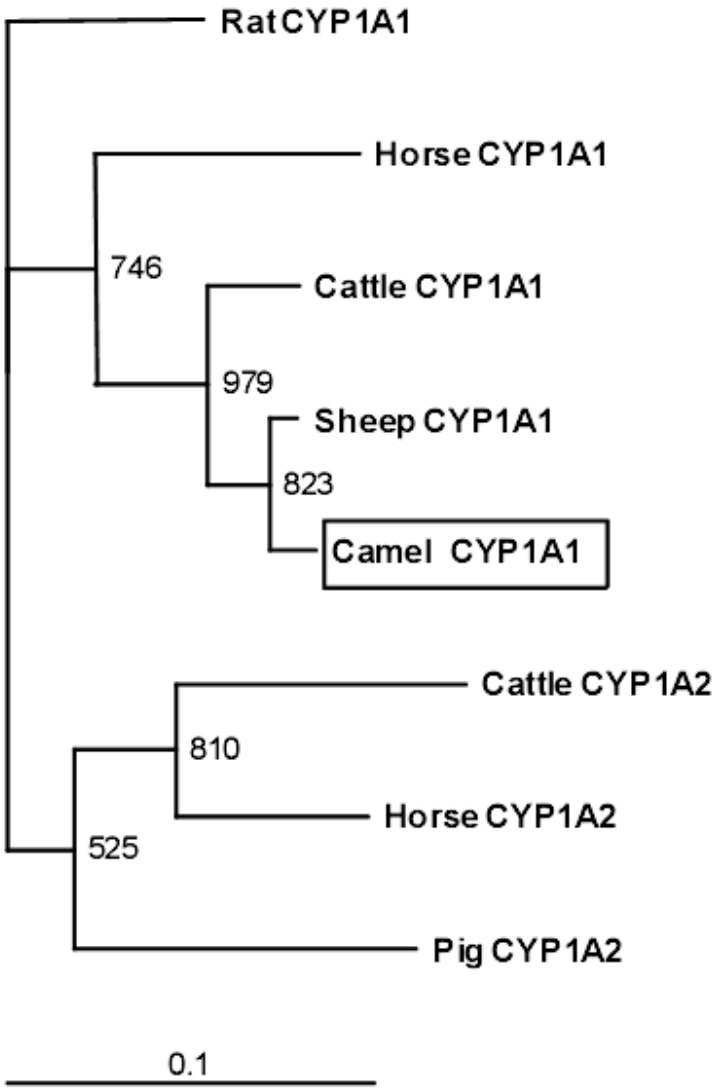


Fig. 21. Phylogenetic analysis of camel and ungulate deduced amino acid sequences of CYP1As

This is a rooted tree for amino acid sequences in the partial region constructed by the Kimura two-parameter model and neighbor H joining method using the PHYLIP program. The numbers on the tree represent local bootstrap values from 1000 replicates. Values less than 100 were removed. Rat CYP1A1 was used as the outgroup species. Bar scale represents number of amino acid substitutions per site.

Tissue distribution of CYP1A1 mRNA in the different tissues of the camel

CYP1A1 mRNA was expressed in all tissues examined including liver except the hump. The highest expression level was observed in lung, liver and tongue compared to other examined tissues (Fig. 22).

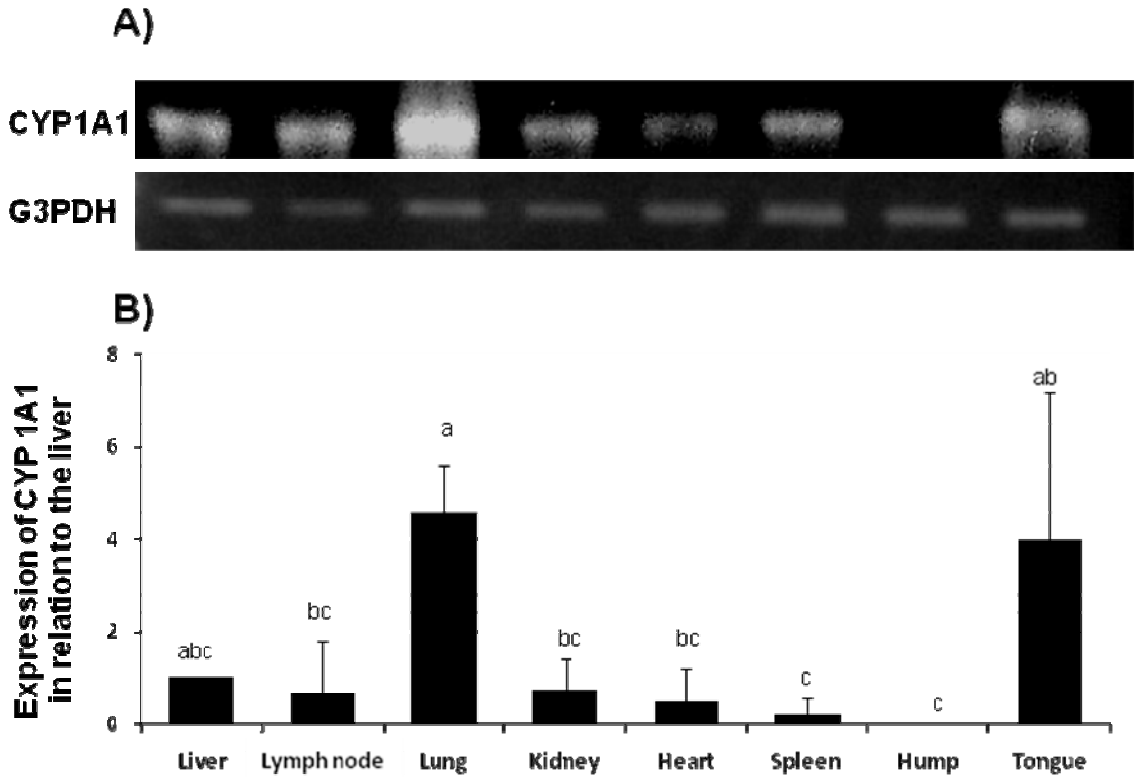


Fig. 22. Tissue distribution of CYP1A1 mRNA in different tissues of the camel

A) DNA bands of CYP1A1; cDNA samples were amplified for CYP1A1 and G3PDH. B) Analysis of the relative amount of each CYP1A1 mRNA by normalization to the corresponding band of G3PDH and calculation in relation to that of the liver. Analysis of the bands was performed using the public domain NIH Image program. This experiment was repeated three times for the three different animals. Different letters indicate significance differences. $P<0.05$.

Discussion

Characterization of CYP1A1 in the camel, which is classified in some parts of the world as a wildlife species and in other parts as a domesticated animal and used as source for both of meat and milk production, is essential for understanding the impact of environmental pollutant exposure on the health status of an exposed population (Hyyti et al., 2001). Since CYP1A1 is a very important isoform as an indicator of environmental pollution and key enzyme for detoxification / activation of xenobiotics, in this chapter, my aim was to characterize CYP1A1 in the camel by studying both the sequence of CYP1A1 and the tissue distribution of CYP1A1 mRNA. I succeeded in amplifying CYP1A1 from a cDNA fragment from the camel liver and obtained a partial sequence of camel CYP1A1. By studying the homology of the isolated camel CYP1A1 with CYP1A isoforms of other ungulates, I found that camel CYP1A1 showed the highest similarity with sheep and cattle CYP1A1 (94%), followed by horse CYP1A1 (87%) and pig CYP1A1 (86%). This result strongly suggests that this clone is camel CYP1A1, not CYP1A2. Although the obtained sequence was only 300 bp, the phylogenetic analysis carried some reliability because the camel clone was located beside sheep and cattle CYP1A1 isoforms.

In the second part of this study, I investigated the tissue distribution of CYP1A1 in different tissues of the camel. The only previous study of the tissue distribution of CYP1A was performed by Raza et al. (1998). They studied the protein expression and CYP1A-dependent activities in the liver, brain, kidney and small intestine only. Here, I examined lung, lymph node, heart, spleen, hump and tongue tissues as well as kidney and liver due to their importance in the metabolism and excretion of xenobiotics. I found that CYP1A1 mRNA was expressed in all tissues except the hump. Interestingly, the lung showed the highest CYP1A1 mRNA expression

compared with the other organs, but the level was similar to that of the tongue. Also the tongues of two of the three camels showed same expression pattern as the lung and liver, but the levels were higher than in the other organs. This may indicate exposure of these animals to AhR ligand, e.g., environmental pollutants or flavonoids, during their lifetime. The expression in the lung was about 4.5 fold higher than that of the liver, and this may have been due to inhalation of pollutants. The most interesting finding was that the tongues of two of the three animals showed high expression levels of CYP1A1, like those of the lung and liver but higher than the other organs in the examined animals. There are very few reports about the expression of CYP1A1 in the tongue either in vivo or in tongue cell culture lines. One of these reports, that of Yang and Raner (2005), who found that both tongue and liver cell lines show the same expression levels of CYP1A1 and CYP1A2 after incubation with β-naphthoflavone as an inducer for CYP1A. A report by Yang et al. (2003) found that the rabbit tongue expresses CYP1A1, CYP2E1 and CYP4A4. I speculated that the rumination of these animals cause repeated exposure to xenobiotics, and this may be one possible means of induction of CYP1A in tongue tissue.

In conclusion, the deduced amino acid sequence of CYP1A1 in the camel showed the highest similarity with those of sheep and cattle (94%). All examined tissues expressed mRNA of camel CYP1A1 except the hump, while the lung showed the highest expression of CYP1A1.

SUMMARY

Meat-producing animals are frequently exposed during their lifetime to a lot of xenobiotics which affect on their biological systems, growth, disease response and lead to changes on the carcass quality. These changes may have some public health impact if people consumed such contaminated meat or meat products. Meat-producing animals have developed enzyme systems which help them to metabolize such xenobiotics. Studying of the profile of the different enzymes used in xenobiotics metabolism may be a good tool to reflect the pre-slaughter exposure to xenobiotics in the meat-producing animals. As many of these enzymes tend to increase upon exposure to xenobiotics, so these enzymes are considered as biomarkers for xenobiotics exposure.

Cytochrome P450 superfamily and other phase II enzymes are considered as major enzyme systems that play important roles in xenobiotics metabolism. Thus, in this thesis, I studied the biological response to xenobiotics in meat-producing animals by studying the characterization of different phase I and II enzymes, mainly CYP1A, UGT1A1 and GSTA1 subfamilies, in these animals.

In chapter I, I investigated the tissue-specific mRNA expression of different cytochrome P450 (CYP) isoforms, UDP glucuronosyl transferase 1A1 (UGT1A1) and glutathione-S-transferase (GSTA1) in the different tissues of cattle using quantitative real-time polymerase chain reaction (qPCR). CYP1A1-like mRNA was expressed in all of the tissues examined including liver, with the highest expression level in kidney. CYP1A2-, 2E1- and 3A4-like mRNAs were only expressed hepatically. Interestingly, significant expression of CYP2B6-like

mRNA was recorded in lung tissue, while CYP2C9-like mRNA was expressed in liver and kidney tissues of the examined cattle. UGT1A1- and GSTA1-like mRNA were expressed in all of the examined tissues, except the mammary glands, and the highest expression levels were recorded in kidney. The high expression of UGT1A1 in lung tissue and GSTA1 in liver tissue was unique to cattle, this has not been reported for rats or mice. The findings of this chapter strongly suggest that the liver, kidneys and lungs of cattle are the major organs contributing to xenobiotic metabolism. Moreover, induction of CYP1A1, UGT1A1 and GSTA1 are considered as good biological biomarkers for pre-slaughter exposure to xenobiotics.

In chapter II, I extended my study to include other growing sources for meat production such as deer and horses. Thus, I investigated and characterized 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. Also, I 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 chapter. In particular, I 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.

In chapter III, I compared the mutagenic activation activity of hepatic microsomes from the three meat-producing animals (cattle, deer and horses) with those of rats as a reference species. In the Ames *Salmonella typhimurium* TA98 assay, the liver microsomes of all examined animals mutagenically activated benzo[*a*]pyrene, an ideal promutagens, in terms of production of

histidine-independent revertant colonies. The microsomes of horses had the highest ability to produce revertant colonies of the examined animals under both low and high substrate concentrations. Inhibition of this mutagenic activity using α-naphthoflavone, anti-rat CYP1A1, anti-rat CYP3A2 and anti-rat CYP2E1 antibodies suggested that this activity was mainly because of CYP1A1 in these animals as well as in rats. The addition of co-factors for two phase II enzymes, microsomal UDP glucuronosyl transferase (UGT) and cytosolic glutathione-S-transferase (GST), reduced the production of the revertant colonies in a concentration-dependent manner. Interestingly, horses had the highest reduction rate among the examined animals, suggesting that phase II enzymes play a great role in producing a state of balance between the bioactivation and detoxification of xenobiotics in these meat-producing animals.

In chapter IV, I elucidated that 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. CYP1A expression and

dependent activities in the treated cells had confirmed that the carotenoid accumulation is, at least in part, a regulator for the inter-species differences in CYP1A expression and activities.

In chapter V, I determined a partial sequence of CYP1A1 in the camel and its phylogenetic position. The deduced amino acid sequence of camel CYP1A1 showed the highest identity 94% with those of sheep and cattle CYP1A1. In a phylogenetic analysis, the camel CYP1A1 isoform was located beside sheep and cattle CYP1A1. When I studied the distribution of camel CYP1A1 mRNA in different tissues, I found that this isoform was expressed in all tissues except the hump. Interestingly, the lungs of all the camels and tongues of two of the three animals showed high expressions of CYP1A1 mRNA, and this may indicate exposure to ligands of aryl hydrocarbon receptor such as environmental pollutants or flavonoids.

In conclusion, in this thesis, I clarified the biological defense systems to xenobiotics in the meat-producing animals. I confirmed the inter-species differences in CYP1A expression and dependent activities. Subsequently, I explained the mechanism of the protection of these animals against the mutagenic activation of promutagens and procarcinogens. Also, I declared a possible cause for the inter-species differences in CYP1A dependent activities and expression. Moreover, I characterized cytochrome P450s and phase II enzymes in some ungulates such as camel, cattle, deer, horses and deer in comparison to rats.

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