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Biochemical characterization of canine cytochrome P450 1A1 variants using recombinant expression

재조합 발현을 이용한 개 cytochrome P450 1A1 변이형의 생화학적 분석

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수의병인생물학 및 예방수의학 (환경위생학) 전공

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Abstract

Biochemical characterization of canine cytochrome P450 1A1 variants using recombinant expression

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Cytochrome P450 1A1 (CYP1A1) is a heme-containing enzyme involved in metabolism of xenobiotics. Although a number of human CYP1A1 have been studied extensively, there are limited studies reported on the characterization of the CYP1A1 in dog. In this study, we isolated, sequenced CYP1A1 cDNA in Sapsaree breed and two kinds of CYP1A1 cDNA sequences, Sap1 and Sap2, were found. Single nucleotide change was found between two sequences, which have Trp in the Sap1 variant and Leu in the Sap2 variant at position 50. The canine CYP1A1 variants were expressed in *Escherichia coli*

and human CYP1A1 was used as control. CYP contents (*i.e.* holoenzyme) and heme contents were higher in the Sap2 variant compared to the contents of the Sap1 variant. However 7-ethoxyresorufin *O*-dealkylation (EROD) activities were reduced in *E.coli* membranes expressing the Sap2 variant. To clarify the impact on the holoenzyme formation and catalytic activity, substitution of codon 50 was conducted. The substitution of codon 50 by isoleucine exhibited a similar CYP content with the Sap2 variant, whereas substitution of other hydrophobic amino acid residues did not increase the level of CYP produced. However, EROD activities revealed that the substitution of codon 50 with hydrophobic amino acid residues such as Val, Ile, and Leu showed lower catalytic activities than those of other hydrophobic amino acid residues. The substitution to residues having polar or charged amino acid such as Lys, Asp and Asn also did not increase in CYP content and catalytic activities of the recombinant enzymes. These findings suggest that the position of codon 50 within canine CYP1A1 affects heme incorporation and enzyme activities.

Keywords: canine CYP1A1, enzyme activity, heme, holoenzyme, enzyme activity, proline-rich region

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List of Abbreviations

Ala-50	Alanine substitution at residue 50 in canine CYP1A1
Asn-50	Asparagine substitution at residue 50 in canine CYP1A1
Asp-50	Aspartic acid substitution at residue 50 in canine CYP1A1
BCA	Bicinchoninic acid
CO	Carbon monoxide
CO_2	Carbon dioxide
CYP1A1	Cytochrome P450 1A1
CYP450	Cytochrome P450
δ - ALA	δ - Aminolevulinic acid
DLPC	1,2-Dilauroyl-sn-glycero-3-phosphocholine
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Ethoxyresorufin
EV	Empty control vector
Hm	Human CYP1A1 Wild-type
Ile-50	Isoleucine substitution at residue 50 in canine CYP1A1
Lys-50	Lysine substitution at residue 50 in canine CYP1A1
ME	β-Mercaptoethanol
Met-50	Methionine substitution at residue 50 in canine CYP1A1

FBS	Fetal bovine serum
NADP+	Nicotinamide adenine dinucleotides
NADPH	Reduced nicotinamide adenine dinucleotide
NPR	NADPH-cytochrome P450 reductase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Phe-50	Phenylalanine substitution at residue 50 in canine CYP1A1
PMSF	Phenylmethylsulfonyl fluoride
PR	Pro-rich
Sap1	Tryptophan substitution at residue 50 in canine CYP1A1
Sap2	Leucine substitution at residue 50 in canine CYP1A1
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel eletrophoresis
SRS-5	Substrate recognition site-5
W46L	Tryptophan to leucine substitution at residue 46 in human CYP1A1
Val-50	Valine substitution at residue 50 in canine CYP1A1

Introduction

Cytochrome P450 (CYP) is a superfamily of enzymes that play an important role in the oxidative metabolism of a wide variety of xenobiotics as well as endogenous compounds (Nelson et al., 1996). CYPs are integral endoplasmic reticulum membrane-anchored proteins, with their *N*-terminus embedded in the membrane and their catalytic domain exposed to the cytosol (Lee et al., 2015).

Among various CYPs, the CYP1A subfamily has a broad affinity for polycyclic aromatic hydrocarbons, heterocyclic amines, endogenous substances and naturally occurring chemicals (Darwish et al., 2010). The CYP1A subfamily consists of two members, CYP1A1 and CYP1A2, and shows strong conservation among species (Martignoni et al., 2006). Endogenous substrates of CYP1A1 include inflammatory mediators such as arachidonic acid, and hormones such as 17β-estradiol and melatonin (Lee et al., 2015). Although most of the P450s are localized in the liver, CYP1A1 is expressed preferentially in the extra-hepatic tissues and are expressed in the liver following induction with polycyclic aromatic hydrocarbons, PAHs (Dey et al., 2001). 7-Ethoxyresorufin *O*-dealkylation (EROD; Burke et al., 1977) have been extensively used to determine the activity of CYP1A1 enzyme.

Genetic polymorphisms within CYPs mainly affect the

metabolism of chemicals that are substrates for those particular enzymes, leading to differences in chemical response (Ingelman-Sundberg et al., 2007; Kirchheiner and Seeringer, 2007). Many allelic variants and several sub-variants have been described for the human CYP1A1 gene (http://www.cypalleles.ki.se/). CYP1A1 genotypes have been associated with various human cancers such as those of the lung, breast, prostate, and ovary (Zhou et al., 2009). Breast cancer is the most common malignancy in women and the mammary gland is a common site for tumor development in bitches. Also, carcinomas of the prostate is a very common condition in men and occurs more frequently in neutered dogs (Dobson JM. 2013).

Dog is an important, widely used species within the pharmaceutical industry for assessing the metabolism, pharmacokinetics, safety, and efficacy of drugs and drug candidates in discovery and development because of their similarities to human anatomy and physiology (Shou et al., 2003; Scherr et al., 2011). In addition, dog pharmacokinetic data along with *in vitro* metabolic data can be very useful for the prediction of human *in vivo* pharmacokinetics and interpretation of toxicity and efficacy results in both species (Mise et al., 2008). A large number of CYP cDNA clones have been isolated, sequenced and extensively studied in humans as well as in rodents and rabbits (http://drnelson.utmem.edu/CytochromeP450.html). In contrast, the number of studies on canine CYP cDNAs is small, even though the dog is extensively used in pharmacology research as well as in drug safety assessment

studies (Tenmizu et al., 2004).

At present, various canine CYP enzymes have been cloned and sequenced include CYP1A1, CYP1A2 (Uchida et al., 1990), CYP2B11 (Graves et al., 1990), CYP2C21, CYP2C41 (Uchida et al., 1990; Blaisdell et al., 1998), CYP2D15 (Sakamoto et al., 1995), CYP2E1 (Lankford et al., 2000), CYP3A12 and CYP3A26 (Ciaccio et al., 1991; Fraser et al., 1997). For almost all these P450s, heterologous expression, functional characterization, and comparison of substrate specificity with corresponding human P450s have been investigated (Mise et al., 2008). Recently, a number of pharmacokinetic studies have performed in canine CYP1A2 (Tenmizu et al., 2004; Tenmizu et al., 2006; Mise et al., 2008; Scherr et al., 2011; Whiterock et al., 2012; Locuson et al., 2015). However, only limited knowledge on functional characterization of canine CYP1A1 is currently available.

In this study, we characterized two canine CYP1A1 variants, Sap1 and Sap2, by using heterologous expression in *Escherichia coli*. A single amino acid difference showed between two variants, which have Trp and Leu at position 50 in the Sap1 variant and the Sap2 variant, respectively. Based on the sequence alignment, codon 50 of canine CYP1A1 is predicted to be located in codon 46 of human CYP1A1. In our laboratory, we recently characterized a human CYP1A1 variant harboring a Gly45Asp substitution by using heterologous expression in *Escherichia coli* and mammalian cells. The Gly45Asp substitution located in a Pro-rich (PR) region leads to a

structural disturbance of CYP1A1, reducing its holoenzyme formation and catalytic activities (Lee et al., 2015). In the present study, our findings suggest that the Sap1 variant apoprotein has a low affinity for its prosthetic heme group but that the Sap1 variant has increased enzymatic activities compared with the Sap2 variant. Based upon site-directed mutagenesis studies, the Sap2 variant's increased capacity for holoenzyme formation and reduced capacity for enzymatic activities may be due to the importance of codon 50 within canine CYP1A1.

Materials and Methods

Chemicals

All chemicals used were of analytical grade or higher and were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

Preparation of lymphocytes

Blood samples were collected from two pedigreed Sapsarees in Cheju National University. Lymphocytes were isolated from 5 mL of whole blood using Lymphoprep (Axis-Shield PoC AS, Oslo) according to the manufacturer's instructions. Isolated cells were washed twice with phosphate-buffered saline (PBS) and then suspended in 1 mL of RPMI 1640 medium containing 2 mg/mL sodium bicarbonate, 10% (*v*/*v*) fetal bovine serum (FBS), and 100 U/mL penicillin. Then, the lymphocytes were treated with 10 nM 2, 3, 7, 8-tetrachlorodibenzodioxin in a humidified 5% CO_2 incubator at 37°C for 24 h.

cDNA cloning and sequencing

Total RNA was extracted by TRIzol® Reagent (Ambion/Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. RNA (1 μg) was used to synthesize cDNA using

M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in the presence of random primers according to the manufacturer's instructions. Based on the nucleotide sequence information of the canine CYP1A1 gene (Accession No. XP_003433938.1), primers for PCR were designed. Full-length cDNAs encoding canine CYP1A1 were initially isolated by PCR with primers listed in Table 1. About 1.6 Kb PCR products were subcloned into pTOP Blunt V2 using TOPcloner™ Blunt core Kit (Enzynomics, Daejeon, South Korea) for sequencing. Sequencing was performed on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequence homology between Boxer (Accession No. XP_003433938.1) and Sapsaree genes was performed with NCBI-BLAST database.

Bacterial constructs

Two open reading frame cDNA CYP1A1 clones were isolated and the cDNA sequences were modified for expression in *E. coli* according to Guo et al. (1994). The second *N*-terminal residue of CYP1A1, Met, was replaced with Ala, and the nucleotide sequences encoding residues 3 - 9 were changed to AT-rich sequence (5′-ATGGCTTCTATGTTTAGACTTTCTATT-3′) without substitution of residues. Each cDNA fragment was inserted into the *Nde*I and *Xba*I restriction sites of pCW-NPR, a human NADPH-cytochrome P450 reductase(NPR)-containing bicistronic expression vector (Parikh et al., 1997).

Bacterial expression

Bacterial harvest and membrane preparation were performed as described previously (Gillam et al., 1993; Guengerich and Martin, 2006). *E. coli* DH5α cells were transformed with the expression constructs and grown overnight at 37°C in Luria - Bertani broth containing 50 μg/mL ampicillin. The overnight culture was inoculated 1:1000 into Terrific Broth medium containing 50 μg/mL ampicillin and 1 mM thiamine. Cultures were incubated at 37°C with shaking at 200 rpm until they attained an OD_{600} of 0.5 - 0.7, then were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (Amresco, Solon, OH) and 0.5 mM δ-aminolevulinic acid (Cayman Chemical, Ann Arbor, MI), a heme precursor ,and cultured for 24 h at 29°C with shaking at 200 rpm.

Culture was then chilled on ice and centrifuged at 3,800 × *g* for 20 min. The cell pellets were washed with PBS, and the cells were weighed and resuspended in 100 mM Tris-acetate buffer, pH 7.6, containing 500 mM sucrose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Lysozyme was added to 0.2 mg/mL, and the suspensions were diluted two-fold with distilled water before incubation on ice for 30 min. The resulting spheroplasts were sedimented at 3,800 × *g* at 4°C for 20 min, and resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (*v*/*v*), and 10 mM β-mercaptoethanol (ME).

Suspensions of spheroplasts were sonicated four times for 20 s each, on ice, and centrifuged at 10,000 × *g* at 4°C for 20 min. Supernatants were centrifuged at 180,000 × *g* at 4°C for 75 min. Sedimented membrane fractions were resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (*v*/*v*), and 10 mM ME. The membrane preparation was stored at −70°C until use.

CYP and heme contents

CYP content was determined by reduced CO difference spectra (Omura and Sato, 1964). Sodium dithionite was added to reduce ferric CYPs. Ferrous-CO CYP complexes were generated by passing CO gas through solutions of the ferrous CYPs. The spectra were collected on a spectrophotometer at room temperature. Heme content was quantified using a pyridine hemochromogen assay and calculated from the difference in absorption between 557 and 575 nm (Schenkman and Jansson, 2006; Sinclair et al., 2001).

Immunoblots

Immunoblots were performed using a primary anti-CYP1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GenDepot, Barker, TX). Blots were developed using a

chemiluminescence reagent kit. Protein concentrations were determined with a BCA protein assay kit (Pierce Biotechnology, Rockford, lL) using bovine serum albumin as a standard.

Site-directed mutagenesis

Site-directed mutagenesis was performed to generate sequence variants of CYPs using an EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, South Korea) according to the manufacturer's instructions. Primers used for mutagenesis are listed in Table 1.

7-Ethoxyresorufin *O*-dealkylation (EROD)

7-ethoxyresorufin (ER) is a CYP1A1 probe substrate. EROD activities were assayed by fluorometric detection of resorufin using excitation and emission wavelengths of 544 and 595 nm, respectively (Chang and Waxman, 2006; Shimada and Yamazaki, 1998). The reaction mixture contained 20 μg of dilauroylphosphatidyl choline, 0.5 - 20 μM ER dissolved in dimethylsulfoxide, and *E. coli* membrane fractions in a total volume of 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4. Following a 5 min pre-incubation at 37°C in a water bath, reactions were initiated by the addition of the NADPH-generation system (final concentrations 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 0.25 IU of yeast glucose 6-phosphate dehydrogenase). Incubations were terminated after 10 min by the

addition of 1 mL ice-cold methanol.

Sequence alignment

To compare the amino acid sequences of CYP1A1s from various species, a multiple-sequence alignment was generated using the ESPript 3.0 alignment program (Robert and Gouet, 2014).

Statistics

All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS 19.0 for Windows (SPSS, Chicago, IL). Differences between sample groups were analyzed using the unpaired Student's *t*-test and one-way analysis of variance followed by the Tukey's *post hoc* test. A *P*-value of < 0.05 was considered significant.

Results

Sapsaree CYP1A1 cDNAs

A number of CYP1A1 cDNA sequences were aligned with Boxer cDNA sequences. Each cDNA contained an open reading frame of 1575 nucleotides, encoding 525 amino acids, including an initiation codon ATG and a termination codon TAG. Based on these alignments, two kinds of CYP1A1 cDNA sequences, Sap1 and Sap2, were found and registered to NCBI (Accession No. KP340900; KP340901). Both the Sap1 and the Sap2 variants have an amino acid change from a Ser codon to a Pro codon at position 522 (TCT to CCT). Additionally, the Sap2 variant differed from Sap1 variant by having an G → T transition at nucleotide 149, thus changing codon 50 from Trp to Leu (TGG to TTG) (Fig. 1).

Expression in *E. coli*

Whole *E. coli* cells expressing Sap1 variant and Sap2 variant as well as their membrane fractions were used for CO-difference spectra (Fig. 2). The cellular CYP content (*i.e.*, holoenzyme) in *E. coli* expressing the Sap2 variant was about 1.7-fold higher than that for the Sap1 variant ($P < 0.05$; Fig. 2A). The membrane fraction expressing the Sap2 variant also had about 1.8-fold higher CYP content than that of the Sap1 variant ($P < 0.05$; Fig. 2B). Based

upon immunoblots, the level of total CYP1A1 protein (apoprotein + holoprotein) in the whole cells and in membrane fractions of *E. coli* expressing two canine CYP1A1 variants was similar to the level in cells and membranes. These observations indicate that the expression of the apoprotein and its incorporation into *E. coli* membranes were not affected by the substitution. *E. coli* transformed with human CYP1A1 WT was used as a positive control. And *E. coli* transformed empty control vector (EV) was used as a negative control, for which CYP content and CYP1A1 protein expression were undetectable.

The heme content in the membrane fractions of *E. coli* expressing the Sap2 variant was 1.64-fold higher than that for the Sap1 variant and about 5-fold higher than in the EV control group ($P < 0.05$; Fig. 2C). The membrane fraction of *E. coli* transformed with EV had a heme level of 0.20 nmol/mg, which may represent endogenous heme levels in the *E. coli* membrane fraction. After deducing EV heme levels from those of Sap1 and Sap2 groups, the heme level of the Sap2 membrane fraction was 1.9-fold higher than that for the Sap1 variant [(0.64 - 0.20)/(1.05 - 0.20)=1.933)], which is similar to CYP level differences in whole *E. coli* cells and membrane fractions (Fig. 2A and 2B). These findings suggest that the Sap2 variant results in an increment of holo-CYP1A1 expression by increasing the heme content of the protein without affecting the expression of CYP1A1 apoprotein. We therefore hypothesize that the position of codon 50 within canine CYP1A1 is an important residue, which alters the ordered framework of the protein structure and

causes an increase of heme incorporation.

Enzyme activities of *E. coli*

Membrane fractions of *E. coli* expressing Sap1 variant and Sap2 variant were used for EROD enzyme assay. In terms of nmol/min/mg protein, EROD activities of the Sap1 variant-expressing membranes exhibited almost equivalent to those of the Sap2 variant-expressing membranes ($P < 0.05$; Fig. 3A). However, catalytic activities (measured in product formed per CYP content) of the Sap2 variant-expressing membranes were 60.3% of the Sap1 counterparts, respectively ($P < 0.05$; Fig. 3B).

Substitution of human CYP1A1

CYP content was measured in *E. coli* expressing the Trp46Leu variant and human CYP1A1 WT protein (Fig. 4A and 4B). The cellular CYP content in *E. coli* expressing the Trp46Leu variant was about 1.2-fold higher than that for the WT ($P < 0.05$; Fig. 4A). The membrane fraction expressing the Trp46Leu variant also had 1.2-fold higher CYP content than that of the WT ($P < 0.05$; Fig. 4B). The total CYP1A1 protein level of the variant was similar to that of WT in the whole cells and membrane fractions.

Membrane fractions of *E. coli* expressing Trp46Leu variant and WT proteins were used for EROD enzyme assay. EROD

activities (measured in product formed per mg protein) of the variant-expressing membranes were similar to those of the WT counterparts, respectively ($P < 0.05$; Figs. 4C). However, EROD activities for the variant-expressing membranes were 80% of those of the WT-expressing membranes in terms of nmol/min/nmol CYP, respectively ($P < 0.05$; Figs. 4D).

Substitution of codon 50

The CYP content was measured in *E. coli* expressing various single-residue variants of codon 50 (Fig. 5 and 7). The expression levels of total CYP1A1 protein were similar among the sample groups analyzed. *E. coli* expressing a small hydrophobic side chain such as Ala had CYP contents that were 80% of that for the Sap1 group ($P < 0.05$; Fig. 5). The CYP contents following substitution with residues having large hydrophobic side chains, Met and Phe, were almost equivalent of that for the Sap1 group. However, substitution to Val, having a hydrophobic side chain, resulted in 1.3-fold increase of the CYP content compared with the Sap1 variant. Additionally, *E. coli* expressing Ile-50 variant, which have similar residue masses to Leu, had CYP content that was higher by 1.6-fold than cells expressing the Sap1 variant and was similar to that of the Sap2 variant ($P < 0.05$; Fig. 5). In contrast, substitution to residues having polar or charged side chains such as Lys, Asp, and Asn caused >40% reduction of CYP content in *E. coli* expressing the Sap1 variant ($P < 0.05$; Fig. 7).

Catalytic activities of the *E. coli* membrane fractions expressing substitution of codon 50

Membrane fractions of *E. coli* expressing two canine CYP1A1 proteins and single-residue variants were used in the enzyme assay. In terms of nmol/min/mg protein, the substitution with hydrophobic amino acid residues did not affect EROD activities ($P < 0.05$; Fig. 6A). However, Val-50, Ile-50, and the Sap2 variant-expressing membranes showed lower EROD activities than those of the other variants in terms of nmol/min/nmol CYP ($P < 0.05$; Fig. 6B).

Compared to the Sap1 and Sap2 variants, the substitution with a polar or a charged amino acid residues resulted in a slight decrease in EROD activities (nmol/min/mg protein) ($P < 0.05$; Fig. 8A). In contrast, the variants having a polar or a charged residues showed similar EROD activities (measured in product formed per CYP content) to those of the Sap2 variant, ($P < 0.05$; Fig. 8B).

Discussion

The current study showed that the Sap2 variant, having leucine at position 50, resulted in an increase of holoenzyme expression compared to the Sap1 variant (Fig. 2). In contrast, the differences exhibited a marked difference in catalytic properties of the recombinant CYP1A1 enzymes of canine. We employed one substrate, ethoxyresorufin (ER), for comparison of the functions in two canine CYP1A1 enzymes. ER is a good substrate for CYP1A1. The EROD activities of the Sap2 variant-expressing membranes were much lower than those of the Sap1 variant-expressing membranes in terms of nmol/min/nmol CYP (Fig. 3B). This is the first study of canine CYP1A1 variants. These findings suggest that the substitution of codon 50 by leucine increased heme incorporation, whereas binding affinity for substrates decreased compared to the Sap1 variant.

The canine CYP1A1 amino acid sequence has a higher identity with human CYP1A1 (82%) than with rodent CYP1A1 (77%), and is 13 amino acid longer than the human orthologs. We also conducted site-directed mutagenesis in human CYP1A1 and showed the same results with canine CYP1A1 (Fig. 4).

Proper folding of apo-CYPs is required for heme insertion, and the heme plays an essential role as a template for final protein folding (Correia et al., 2011). The difference between two canine

CYP1A1 sequences was a single amino acid at position 50, which is predicted to be located in codon 46 of human CYP1A1. In human CYP1A1, the PR region has the sequence PPGPWGWPLIGH, where the seven residue is Trp46. In nearly every microsomal CYP molecule, a Pro-rich (PR) region is present following an *N*-terminal signal anchor sequence and a short hydrophilic linker sequence (Williams et al., 2000). The PR region has an important role in the proper folding of various CYP enzymes, especially prior to heme binding (Lee et al., 2015).

A molecular model of the PR region and adjacent structural elements shows that the PR region appears not to be directly involved in heme binding, but indirectly interacts with heme through β-sheet 1 and SRS-5 segments (Lee et al., 2015). Given the critical functions of the PR region's initial residues in holo-CYP1A1 formation, a Trp46Leu substitution in close proximity may have an influence on those residues' structural role in holo-CYP1A1 formation.

Arg77 appears to form a hydrogen bond with Trp46 and to be one of the key residues of β-strand 1-1 that is responsible for interactions with residues of the PR region (Fig. 9). The residue is likely to play an important role in the formation of holo-CYP1A1 enzyme (Lee et al., 2015). The potential change of interactions between Trp46 and Arg77, induced by Trp46Leu substitution, may affect holo-CYP1A1 enzyme formation.

To evaluate the effects of the substitution of amino acid

residue at position 50 in canine CYP1A1, site-directed mutagenesis was conducted. The level of holoenzyme formation on the substitution by Ile was equivalent to that of Sap2 variant having side chain of similar mass, Leu (Fig. 5). In contrast, substitution of codon 50 with residues having other hydrophobic chains resulted in similar levels of holoenzyme formation for the Sap1 variant and decreased levels for the Sap2 variant (Fig. 5). The substitution of residue with a polar and charged side chain exhibited decreased levels of CYP content compared to the Sap1 variant (Fig. 7). It is thus possible that the amino acid residue at position 50, which had Leu and Ile, is important for efficient anchoring of the canine CYP1A1 proteins in the endoplasmic reticulum membrane.

In the catalytic activities, *E. coli* membranes expressing hydrophobic amino acid residues had similar activities of EROD in terms of nmol/min/mg protein (Fig. 6A). On the other hand, the substitution of codon 50 with residues, such as Ile and Val, had lower levels of enzyme activities (nmol/min/nmol CYP) than those of the Sap1 variant-expressing membranes (Fig. 6B). In case of *E. coli* membranes having polar and charged amino acid residues, the levels of the enzyme activities (measured in product formed per CYP content) much lower than those of two canine CYP1A1 variants-expressing membranes (Fig. 8). Based upon these findings, we suggest that residues having Val, Ile, and Leu alter the ordered framework of the protein, affect interaction with substrate recognition site, and lead to a decrease of binding affinity for substrates.

Taken together, these findings suggest that codon 50 in canine CYP1A1 substitution leads to a structural disturbance of CYP1A1, affecting its holoenzyme formation and catalytic activities. It is proposed that the variant having Val, Ile and Leu may have reduced CYP1A1 activity, compared with the Sap1 variant, in various canine tissues and that an individual carrying the variant allele may have different susceptibility to adverse health effects of environmental chemicals that are metabolized via CYP1A1-dependent pathways. Further studies are warranted to extend our understanding of the importance of the polymorphism in environmental health, especially with respect to the influence of the polymorphism on gene expression and regulation, as well as to the association between the polymorphism and the incidence of environmentally associated diseases.

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Figures

Fig. 1

Sequence comparison of CYP1A1s from various animal species. The different species and associated accession numbers for CYP1A1s are as follows; *C. lupus* (XP_003433938.1), *H. sapiens* (NP_000490.1), *P. troglodytes* (XP_001137654.1), *B. taurus* (XP_005222075.1), *M. mulatta* (NP_001035328.1), *M. musculus* (NP_001129531.1), and *R. norvegicus* (NP_036672.2). Conserved residues are in blue boxes; identical residues are shown with a red background, similar residues in red typeface. Different residue between two canine CYP1A1 variants (codon 50) are indicated by a black triangle.

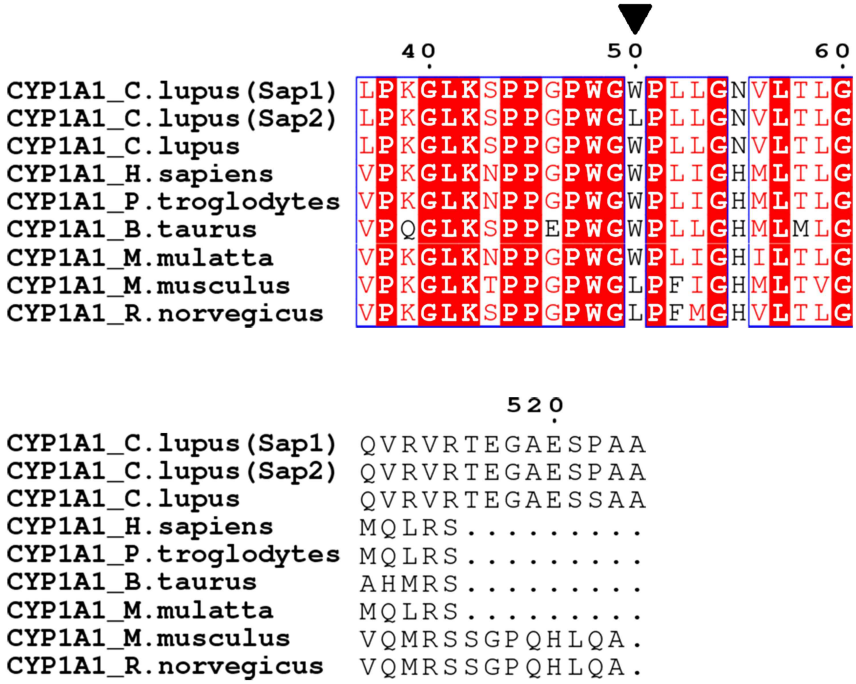

40
50
60
CYP1A1_C.lupus(Sap1) LPKGLKSPPGPWGWPLLGNVLTLG
CYP1A1_C.lupus(Sap2) LPKGLKSPPGPWGLPLLGNVLTLG
CYP1A1_C.lupus LPKGLKSPPGPWGWPLLGNVLTLG
CYP1A1_H.sapiens VPKGLKNPPGPWGWPLIGHMLTLG
CYP1A1_P.troglodytes VPKGLKNPPGPWGWPLIGHMLTLG
CYP1A1_B.taurus VPQGLKSPPEPWGWPLLGHMLMLG
CYP1A1_M.mulatta VPKGLKNPPGPWGWPLIGHILTLG
CYP1A1_M.musculus VPKGLKTPPGPWGLPFIGHMLTVG
CYP1A1_R.norvegicus VPKGLKSPPGPWGLPFMGHVLTLG
520
CYP1A1_C.lupus(Sap1) QVRVRTEGAESPAA
CYP1A1_C.lupus(Sap2) QVRVRTEGAESPAA
CYP1A1_C.lupus QVRVRTEGAESSAA
CYP1A1_H.sapiens MQLRS.....
CYP1A1_P.troglodytes MQLRS.....
CYP1A1_B.taurus AHMRS.....
CYP1A1_M.mulatta MQLRS.....
CYP1A1_M.musculus VQMRSSGPQHLQA.
CYP1A1_R.norvegicus VQMRSSGPQHLQA.

Fig. 2

Expression of two canine CYP1A1 variants. *E. coli* cells were transformed either with pCW-NPR vector including two canine CYP1A1 variants or human CYP1A1 WT cDNAs (Hm), or with EV. Total CYP contents were quantified using reduced CO-difference spectra in (A) whole cells and (B) the membrane fractions. Lower insets represent total CYP1A1 protein expression in whole cells and membrane fractions, which were assessed by immunoblotting. (C) Heme contents were determined in the membrane fractions. Each bar represents the mean ± SD of six independent samples. Different lower-case letters indicate significant differences in each panel ($P < 0.05$, Tukey's *post hoc* test).

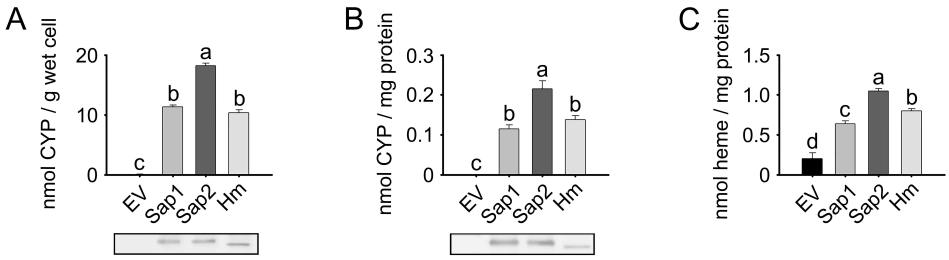

A
nmol CYP / g wet cell
20
10
0
c
b
a
b
EV
Sap1
Sap2
Hm
B
nmol CYP / mg protein
0.3
0.2
0.1
0
c
b
a
b
EV
Sap1
Sap2
Hm
C
nmol heme / mg protein
1.5
1.0
0.5
0
d
c
a
b
EV
Sap1
Sap2
Hm

Fig. 3

Catalytic activities of two canine CYP1A1 variants. (A) EROD activities (measured in product formed per mg protein) of *E. coli* membrane fractions expressing two canine CYP1A1 variants and human CYP1A1 WT. (B) EROD activities (measured in product formed per CYP content) in *E. coli* membrane fractions expressing two canine CYP1A1 variants and human CYP1A1 WT. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of six independent samples ($P < 0.05$, Tukey's *post hoc* test).

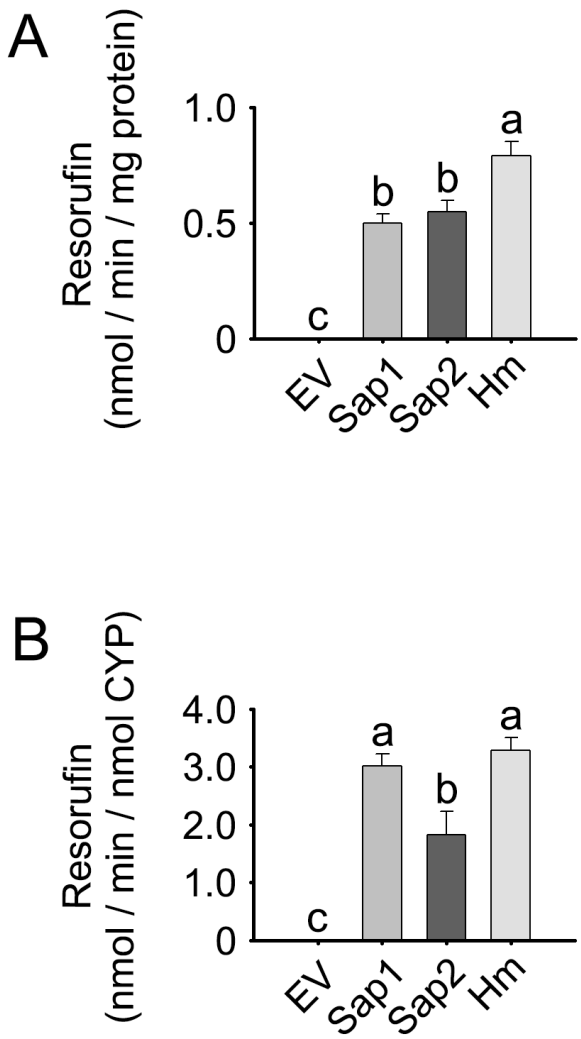

A
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
c
b
b
a
EV
Sap1
Sap2
Hm
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
a
EV
Sap1
Sap2
Hm

Fig. 4

CYP contents and CYP1A1 activities in *E. coli* expressing human CYP1A1 WT and its Trp46Leu variant. CYP contents in (A) whole cells and (B) the membrane fractions. (C) EROD activities (measured in product formed per mg protein) of *E. coli* membrane fractions expressing the variant and WT proteins. (D) EROD activities (measured in product formed per CYP content) of *E. coli* membrane fractions expressing the variant and WT proteins. Detection of total CYP1A1 protein was performed with immunoblots of whole cells and membrane fractions of *E. coli*. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

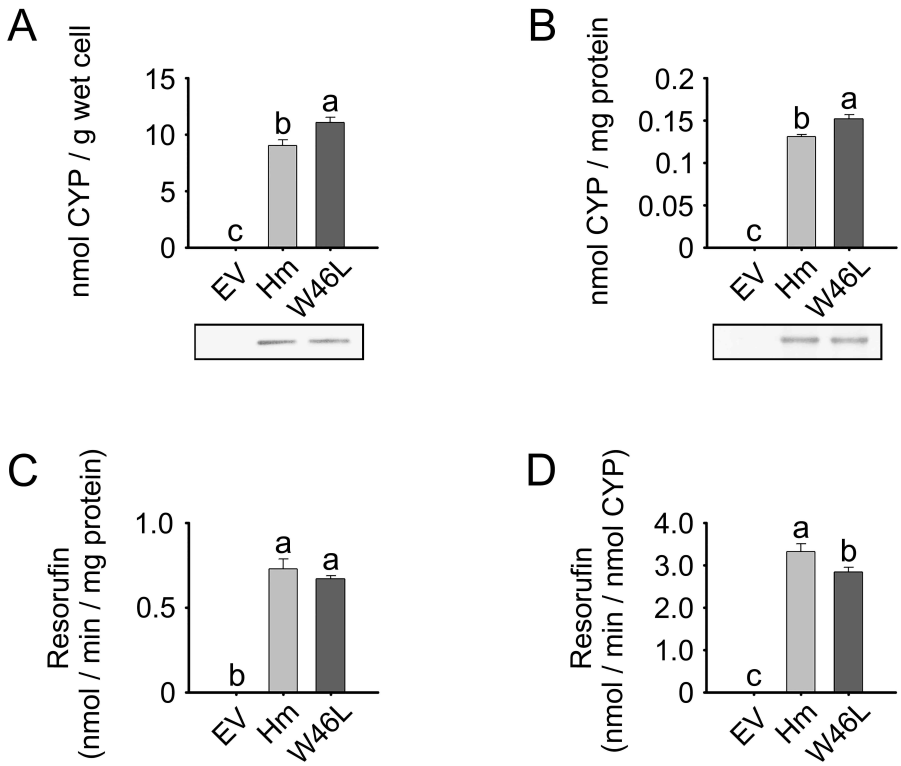

A
nmol CYP / g wet cell
15
10
5
0
c
b
a
EV
Hm
W46L
B
nmol CYP / mg protein
0.2
0.15
0.1
0.05
0
c
b
a
EV
Hm
W46L
C
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
b
a
a
EV
Hm
W46L
D
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
EV
Hm
W46L

Fig. 5

CYP contents for two canine CYP1A1 variants and single-residue variants, having hydrophobic amino acid of codon 50. Two canine CYP1A1 proteins and hydrophobic amino acid residue variants are arranged in order of small to large masses of substituting residues in (A) whole cells and (B) the membrane fractions. Total CYP1A1 protein expression was analyzed with immunoblots. Each bar represents the mean ± SD of four independent samples. Different lower-case letters above the bars indicate a significant difference among the groups ($P < 0.05$, Tukey's *post hoc* test).

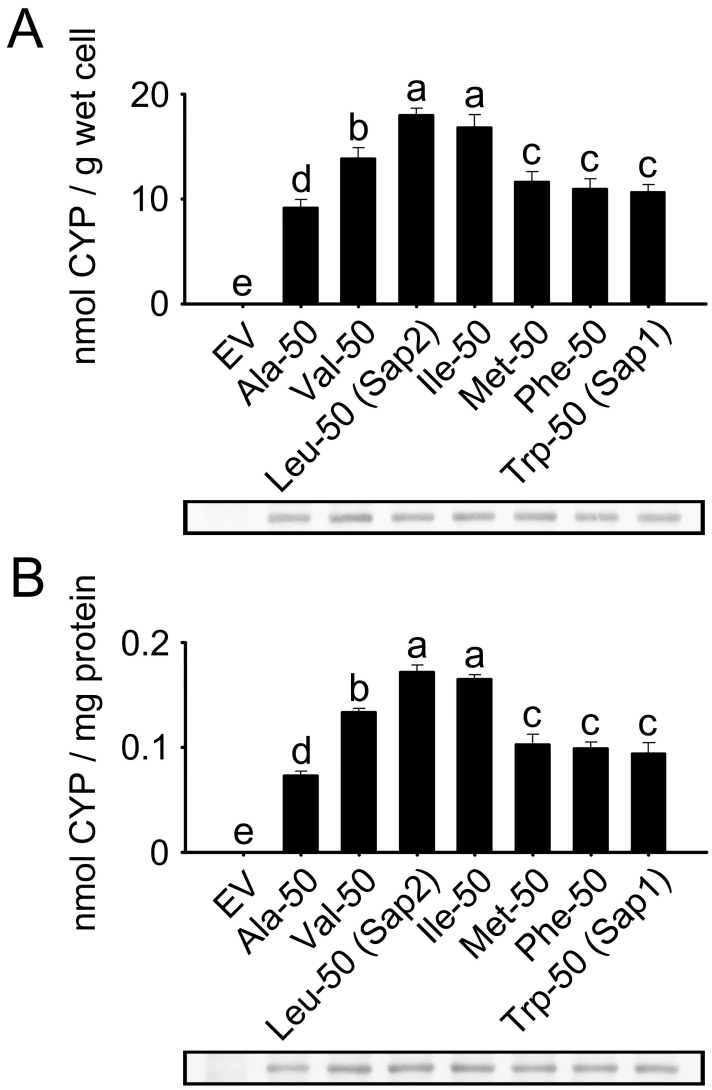

A
nmol CYP / g wet cell
20
10
0
e
d
b
a
a
c
c
c
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)
B
nmol CYP / mg protein
0.2
0.1
0
e
d
b
a
a
c
c
c
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)

Fig. 6

Enzyme activities in *E. coli* membrane fractions expressing two canine CYP1A1 variants and hydrophobic amino acid residue variants of codon 50. (A, B) EROD activities of *E. coli* membrane fractions. A, nmol/min/mg protein; B, nmol/min/nmol CYP. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

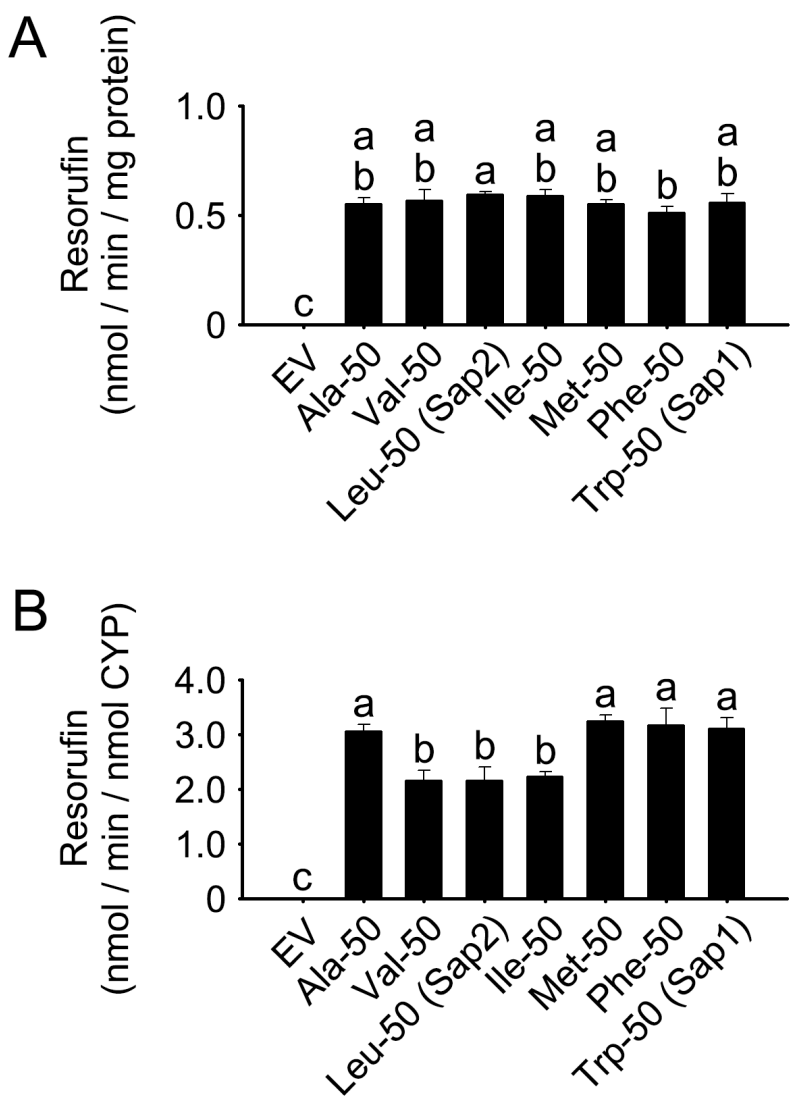

A
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
c
a
b
a
b
a
a
b
a
b
b
a
b
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
b
b
a
a
a
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)

Fig. 7

CYP contents in *E. coli* expressing polar or charged amino acid residue variants of codon 50. (A) Whole cells and (B) the membrane fractions. *E. coli* cells were transformed either with pCW-NPR vector harboring cDNAs or with EV. Total CYP1A1 protein expression was analyzed with immunoblots. Each bar represents the mean ± SD of four independent samples. Different lower-case letters above the bars indicate a significant difference among the groups ($P < 0.05$, Tukey's *post hoc* test).

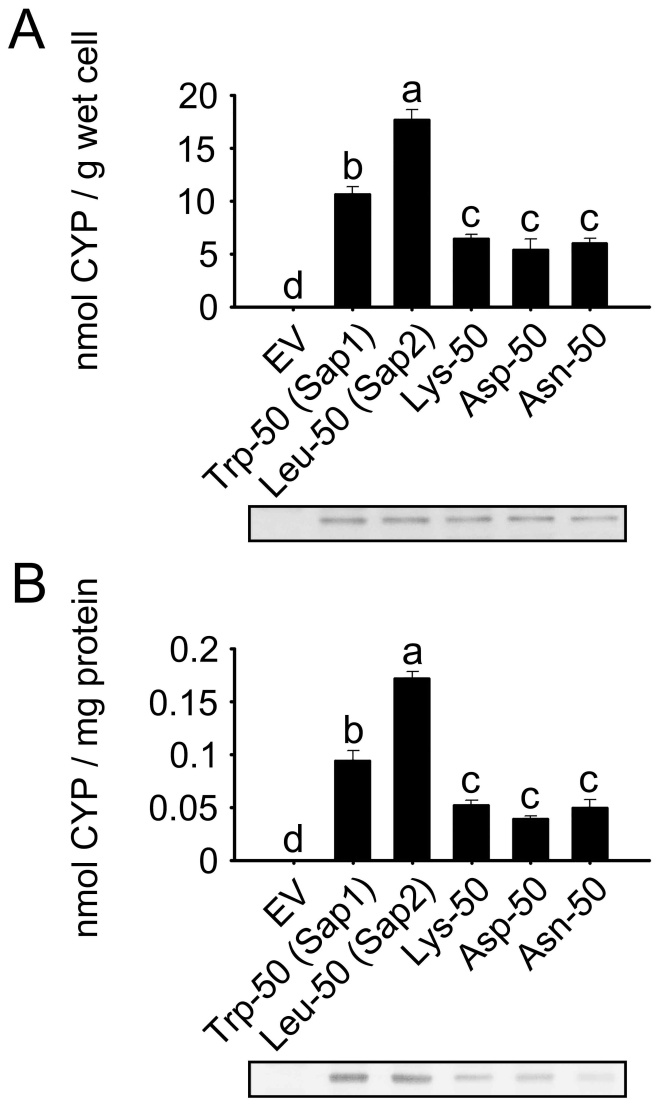

A
nmol CYP / g wet cell
20
15
10
5
0
a
b
c
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50
B
nmol CYP / mg protein
0.2
0.15
0.1
0.05
0
a
b
c
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50

Fig. 8

Catalytic activities in *E. coli* membrane fractions expressing two canine CYP1A1 variants and polar or charged amino acid residue variants of codon 50. (A, B) EROD activities of *E. coli* membrane fractions. A, nmol/min/mg protein; B, nmol/min/nmol CYP. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

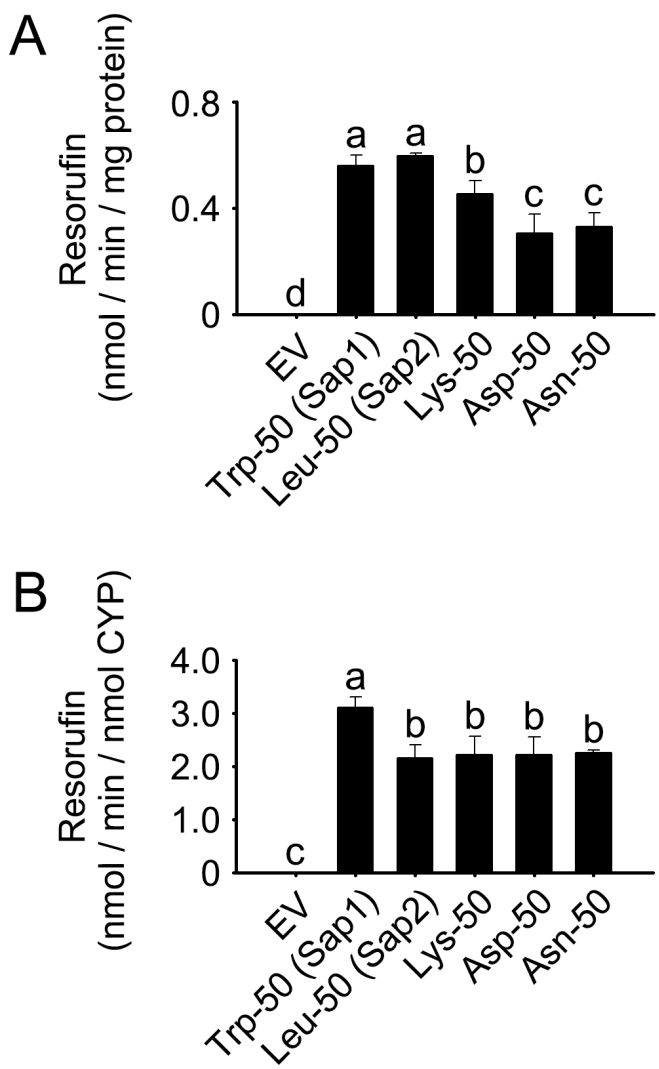
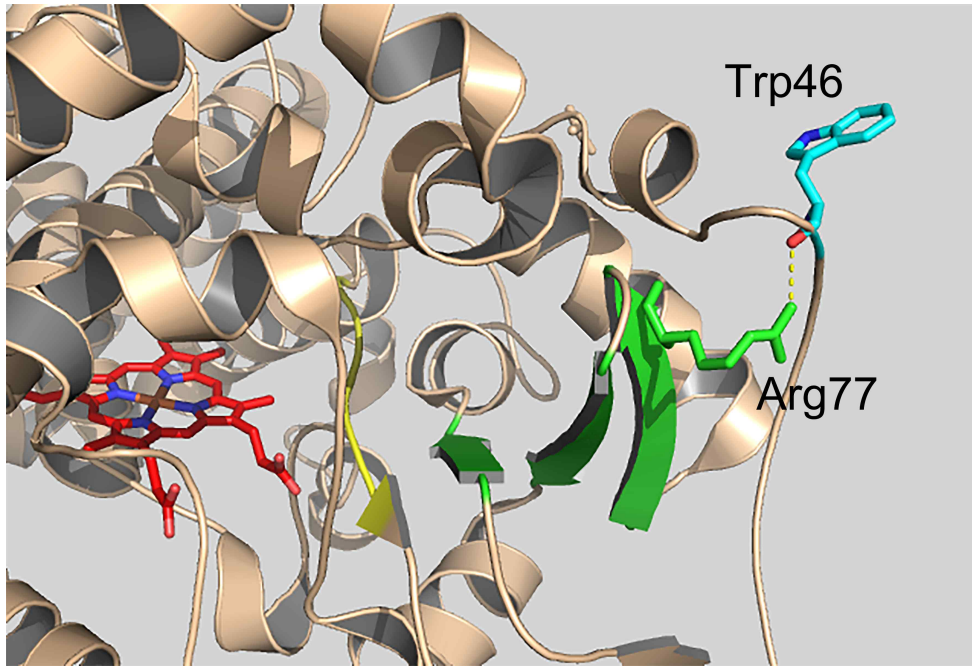

A
Resorufin
(nmol / min / mg protein)
0.8
0.4
0
a
a
b
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
a
b
b
b
b
c
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50

Fig. 9

A three-dimensional structure of CYP1A1 (PDB ID: 4I8 V). A ribbon model of the PR region harboring Trp46 and adjacent structural segments such as β-sheet 1, β-strand 2-2, SRS-5, and heme.


Trp46
Arg77

Table

Table 1. Oligonucleotide primer sequences used for cDNA cloning, *N*-terminal modification, and site-directed mutagenesis. [a]Coding sequences corresponding to the mutated amino acids are underlined and in bold.

	Primer	Oligonucleotide sequence (5' to 3')[a]	Description
	CYP1A1-F	CAG CTG TCT TGA GGT CTC TAC GC	Forward primer for cDNA cloning of canine CYP1A1
	CYP1A1-R	ACC CAG ACA GGC CAG GTA GAC AG	Reverse primer for cDNA cloning of canine CYP1A1
	N-ter-F	CG CTG GTG CAT ATG GCT TCT ATG TTT AGA CTT TCT ATT CCC	Forward primer for *N*-terminal modification of canine CYP1A1
	N-ter-R	GCT CTA GAC TAG GCT GCA GGG CTC	Reverse primer for *N*-terminal modification of canine CYP1A1
	Ala50-F	G GGC **GCT** CCC CTG CTC GGG AAC	Forward primer for site-directed mutagenesis of coding sequence of codon 50 into a Ala
	Ala50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site-directed mutagenesis of coding sequence of codon 50 into a Ala

Canine CYP1A1	Phe50-F	G GGC **TTT** CCC CTG CTC GGG AAC	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Phe
	Phe50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Phe
	Val50-F	G GGC **GTG** CCC CTG CTC GGG AAC	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Val
	Val50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Val
	Met50-F	GC **ATG** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Met
	Met50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Met
	Ile50-F	GC **ATT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Ile
	Ile50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Ile
	Lys50-F	GC **AAG** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Lys
	Lys50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Lys
	Asp50-F	GC **GAT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Asp
	Asp50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Asp

	Asn50-F	GC **AAT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Asn
	Asn50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Asn
Human CYP1A1	W46L-F	C **TTA** CCT CTG ATT GGG CAC ATG	Forward primer for site—directed mutagenesis of coding sequence of Trp46 into a Leu
	W46L-R	CCC CAT GGC CCT GGT GGA TTC	Reverse primer for site—directed mutagenesis of coding sequence of Trp46 into a Leu

국문 초록

재조합 발현을 이용한 개 cytochrome P450 1A1 변이형의 생화학적 분석

Cytochrome P450 (CYP)는 heme-thiolate 효소로서 대부분의 생물 종에서 발견되며, 포유동물에서 약물, 생체 내 물질(endogenous substances), 그리고 생체 외 물질(xenobiotics)의 최초대사와 관련 되어 있다. CYP는 주로 세포 내 소포체(endoplasmic reticulum)에 존재한다. CYP1A1은 간 외 조직, 주로 폐 조직에서 매우 높게 발현되며, 환경 발암물질(environmental carcinogen)과 환경 호르몬의 대사에 매우 중요한 역할을 담당하고 있다. 인간 CYP1A1 유전자는 다형성을 나타내며, 소수의 단일 염기서열다형성이 보고 및 연구 되었다. 하지만 개 CYP1A1 유전자의 다형성에 대한 연구는 아직 보고된 바 없다.

본 연구에서는 삽살개의 cDNA 서열을 분석하여 2종류의 CYP1A1 변이형을 발견하였고, 이를 각각 Sap1과 Sap2라고 명명하였다. 2종류의 CYP1A1 변이형은 50번째 아미노산 서열에서 차이를 보였다. Sap1은 50번째 아미노산에 Trp이 위치하였고, Sap2는 Leu이 위치하였다. 2종류의 CYP1A1 변이형은 대장균 세포 내에서의 CYP1A1 아포효소(apoenzyme)의 전사, 번역 효율에 영향을 주지 않았으며, 대장균 세포막에 CYP1A1 단백질이 결합되는 정도에도 영향을 주지 않았다. 그러나 Sap1의 CYP 완전효소(holoenzyme)의 양은 Sap2에 비해 낮은 것으로 나타났는데, 이것은 Sap1 변이형 단백질이 heme기가 결여된 무기능 단

백질 형태로 존재한다는 것을 시사한다. 하지만, 제 기능이 유지된 완전 효소 양을 기준으로 하여 Sap1과 Sap2 단백질의 7-에톡시레소루핀-*O*-디에틸라아제(EROD) 활성을 비교할 경우, Sap2의 활성이 Sap1의 활성에 비해 낮았다. 이 결과를 토대로, 50번째 아미노산의 변이는 단백질 구조를 변화시키고 heme기의 결합에 영향을 미친다는 것이 예상 되었다.

Site-directed mutagenesis 기술은 CYP1A1의 구조와 기능의 상관관계를 규명하고 중요한 아미노산 서열을 알아내는데 매우 유용하게 사용되었다. CYP1A1의 구조 내 상호작용의 특징을 설명하기 위해 50번째 아미노산을 다른 아미노산으로 치환하여 동일한 실험을 수행하였다. 그 결과 50번째 아미노산 위치에 잔기의 분자량이 40-60 정도 되는 소수성 아미노산인 Val, Leu, Ile이 위치하는 경우 CYP 단백질의 heme기 결합 능력이 증가하는 양상을 보였다. 반면 7-에톡시레소루핀-*O*-디에틸라아제 활성은 감소하는 양상을 보였다.

이러한 결과는 50번째 아미노산의 변이가 CYP 단백질의 heme기의 유입(heme incorporation)과 효소 활성에 영향을 줌으로써, 기능 변화를 유발한다는 것을 시사한다.

주요어: Cytochrome P450 1A1 (CYP1A1), Canine CYP1A1, heme기, 완전효소 (holoenzyme), 에톡시레소루핀-*O*-디에틸라아제 (EROD)

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수의학석사학위논문

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Abstract

Biochemical characterization of canine cytochrome P450 1A1 variants using recombinant expression

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Cytochrome P450 1A1 (CYP1A1) is a heme-containing enzyme involved in metabolism of xenobiotics. Although a number of human CYP1A1 have been studied extensively, there are limited studies reported on the characterization of the CYP1A1 in dog. In this study, we isolated, sequenced CYP1A1 cDNA in Sapsaree breed and two kinds of CYP1A1 cDNA sequences, Sap1 and Sap2, were found. Single nucleotide change was found between two sequences, which have Trp in the Sap1 variant and Leu in the Sap2 variant at position 50. The canine CYP1A1 variants were expressed in *Escherichia coli*

and human CYP1A1 was used as control. CYP contents (*i.e.* holoenzyme) and heme contents were higher in the Sap2 variant compared to the contents of the Sap1 variant. However 7-ethoxyresorufin *O*-dealkylation (EROD) activities were reduced in *E.coli* membranes expressing the Sap2 variant. To clarify the impact on the holoenzyme formation and catalytic activity, substitution of codon 50 was conducted. The substitution of codon 50 by isoleucine exhibited a similar CYP content with the Sap2 variant, whereas substitution of other hydrophobic amino acid residues did not increase the level of CYP produced. However, EROD activities revealed that the substitution of codon 50 with hydrophobic amino acid residues such as Val, Ile, and Leu showed lower catalytic activities than those of other hydrophobic amino acid residues. The substitution to residues having polar or charged amino acid such as Lys, Asp and Asn also did not increase in CYP content and catalytic activities of the recombinant enzymes. These findings suggest that the position of codon 50 within canine CYP1A1 affects heme incorporation and enzyme activities.

Keywords: canine CYP1A1, enzyme activity, heme, holoenzyme, enzyme activity, proline-rich region

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List of Abbreviations

Ala-50	Alanine substitution at residue 50 in canine CYP1A1
Asn-50	Asparagine substitution at residue 50 in canine CYP1A1
Asp-50	Aspartic acid substitution at residue 50 in canine CYP1A1
BCA	Bicinchoninic acid
CO	Carbon monoxide
CO_2	Carbon dioxide
CYP1A1	Cytochrome P450 1A1
CYP450	Cytochrome P450
δ - ALA	δ - Aminolevulinic acid
DLPC	1,2-Dilauroyl-sn-glycero-3-phosphocholine
E.coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid
ER	Ethoxyresorufin
EV	Empty control vector
Hm	Human CYP1A1 Wild-type
Ile-50	Isoleucine substitution at residue 50 in canine CYP1A1
Lys-50	Lysine substitution at residue 50 in canine CYP1A1
ME	β-Mercaptoethanol
Met-50	Methionine substitution at residue 50 in canine CYP1A1

FBS	Fetal bovine serum
NADP+	Nicotinamide adenine dinucleotides
NADPH	Reduced nicotinamide adenine dinucleotide
NPR	NADPH-cytochrome P450 reductase
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
Phe-50	Phenylalanine substitution at residue 50 in canine CYP1A1
PMSF	Phenylmethylsulfonyl fluoride
PR	Pro-rich
Sap1	Tryptophan substitution at residue 50 in canine CYP1A1
Sap2	Leucine substitution at residue 50 in canine CYP1A1
SD	Standard deviation
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel eletrophoresis
SRS-5	Substrate recognition site-5
W46L	Tryptophan to leucine substitution at residue 46 in human CYP1A1
Val-50	Valine substitution at residue 50 in canine CYP1A1

Introduction

Cytochrome P450 (CYP) is a superfamily of enzymes that play an important role in the oxidative metabolism of a wide variety of xenobiotics as well as endogenous compounds (Nelson et al., 1996). CYPs are integral endoplasmic reticulum membrane-anchored proteins, with their *N*-terminus embedded in the membrane and their catalytic domain exposed to the cytosol (Lee et al., 2015).

Among various CYPs, the CYP1A subfamily has a broad affinity for polycyclic aromatic hydrocarbons, heterocyclic amines, endogenous substances and naturally occurring chemicals (Darwish et al., 2010). The CYP1A subfamily consists of two members, CYP1A1 and CYP1A2, and shows strong conservation among species (Martignoni et al., 2006). Endogenous substrates of CYP1A1 include inflammatory mediators such as arachidonic acid, and hormones such as 17β-estradiol and melatonin (Lee et al., 2015). Although most of the P450s are localized in the liver, CYP1A1 is expressed preferentially in the extra-hepatic tissues and are expressed in the liver following induction with polycyclic aromatic hydrocarbons, PAHs (Dey et al., 2001). 7-Ethoxyresorufin *O*-dealkylation (EROD; Burke et al., 1977) have been extensively used to determine the activity of CYP1A1 enzyme.

Genetic polymorphisms within CYPs mainly affect the

metabolism of chemicals that are substrates for those particular enzymes, leading to differences in chemical response (Ingelman-Sundberg et al., 2007; Kirchheiner and Seeringer, 2007). Many allelic variants and several sub-variants have been described for the human CYP1A1 gene (http://www.cypalleles.ki.se/). CYP1A1 genotypes have been associated with various human cancers such as those of the lung, breast, prostate, and ovary (Zhou et al., 2009). Breast cancer is the most common malignancy in women and the mammary gland is a common site for tumor development in bitches. Also, carcinomas of the prostate is a very common condition in men and occurs more frequently in neutered dogs (Dobson JM. 2013).

Dog is an important, widely used species within the pharmaceutical industry for assessing the metabolism, pharmacokinetics, safety, and efficacy of drugs and drug candidates in discovery and development because of their similarities to human anatomy and physiology (Shou et al., 2003; Scherr et al., 2011). In addition, dog pharmacokinetic data along with *in vitro* metabolic data can be very useful for the prediction of human *in vivo* pharmacokinetics and interpretation of toxicity and efficacy results in both species (Mise et al., 2008). A large number of CYP cDNA clones have been isolated, sequenced and extensively studied in humans as well as in rodents and rabbits (http://drnelson.utmem.edu/CytochromeP450.html). In contrast, the number of studies on canine CYP cDNAs is small, even though the dog is extensively used in pharmacology research as well as in drug safety assessment

studies (Tenmizu et al., 2004).

At present, various canine CYP enzymes have been cloned and sequenced include CYP1A1, CYP1A2 (Uchida et al., 1990), CYP2B11 (Graves et al., 1990), CYP2C21, CYP2C41 (Uchida et al., 1990; Blaisdell et al., 1998), CYP2D15 (Sakamoto et al., 1995), CYP2E1 (Lankford et al., 2000), CYP3A12 and CYP3A26 (Ciaccio et al., 1991; Fraser et al., 1997). For almost all these P450s, heterologous expression, functional characterization, and comparison of substrate specificity with corresponding human P450s have been investigated (Mise et al., 2008). Recently, a number of pharmacokinetic studies have performed in canine CYP1A2 (Tenmizu et al., 2004; Tenmizu et al., 2006; Mise et al., 2008; Scherr et al., 2011; Whiterock et al., 2012; Locuson et al., 2015). However, only limited knowledge on functional characterization of canine CYP1A1 is currently available.

In this study, we characterized two canine CYP1A1 variants, Sap1 and Sap2, by using heterologous expression in *Escherichia coli*. A single amino acid difference showed between two variants, which have Trp and Leu at position 50 in the Sap1 variant and the Sap2 variant, respectively. Based on the sequence alignment, codon 50 of canine CYP1A1 is predicted to be located in codon 46 of human CYP1A1. In our laboratory, we recently characterized a human CYP1A1 variant harboring a Gly45Asp substitution by using heterologous expression in *Escherichia coli* and mammalian cells. The Gly45Asp substitution located in a Pro-rich (PR) region leads to a

structural disturbance of CYP1A1, reducing its holoenzyme formation and catalytic activities (Lee et al., 2015). In the present study, our findings suggest that the Sap1 variant apoprotein has a low affinity for its prosthetic heme group but that the Sap1 variant has increased enzymatic activities compared with the Sap2 variant. Based upon site-directed mutagenesis studies, the Sap2 variant's increased capacity for holoenzyme formation and reduced capacity for enzymatic activities may be due to the importance of codon 50 within canine CYP1A1.

Materials and Methods

Chemicals

All chemicals used were of analytical grade or higher and were purchased from Sigma-Aldrich (St. Louis, MO) unless specified.

Preparation of lymphocytes

Blood samples were collected from two pedigreed Sapsarees in Cheju National University. Lymphocytes were isolated from 5 mL of whole blood using Lymphoprep (Axis-Shield PoC AS, Oslo) according to the manufacturer's instructions. Isolated cells were washed twice with phosphate-buffered saline (PBS) and then suspended in 1 mL of RPMI 1640 medium containing 2 mg/mL sodium bicarbonate, 10% (*v*/*v*) fetal bovine serum (FBS), and 100 U/mL penicillin. Then, the lymphocytes were treated with 10 nM 2, 3, 7, 8-tetrachlorodibenzodioxin in a humidified 5% CO_2 incubator at 37°C for 24 h.

cDNA cloning and sequencing

Total RNA was extracted by TRIzol® Reagent (Ambion/Life Technologies, Carlsbad, CA) according to the instructions of the manufacturer. RNA (1 μg) was used to synthesize cDNA using

M-MLV reverse transcriptase (Invitrogen, Carlsbad, CA) in the presence of random primers according to the manufacturer's instructions. Based on the nucleotide sequence information of the canine CYP1A1 gene (Accession No. XP_003433938.1), primers for PCR were designed. Full-length cDNAs encoding canine CYP1A1 were initially isolated by PCR with primers listed in Table 1. About 1.6 Kb PCR products were subcloned into pTOP Blunt V2 using TOPcloner™ Blunt core Kit (Enzynomics, Daejeon, South Korea) for sequencing. Sequencing was performed on a 3730xl DNA analyzer (Applied Biosystems, Foster City, CA). The sequence homology between Boxer (Accession No. XP_003433938.1) and Sapsaree genes was performed with NCBI-BLAST database.

Bacterial constructs

Two open reading frame cDNA CYP1A1 clones were isolated and the cDNA sequences were modified for expression in *E. coli* according to Guo et al. (1994). The second *N*-terminal residue of CYP1A1, Met, was replaced with Ala, and the nucleotide sequences encoding residues 3 - 9 were changed to AT-rich sequence (5′-ATGGCTTCTATGTTTAGACTTTCTATT-3′) without substitution of residues. Each cDNA fragment was inserted into the *Nde*I and *Xba*I restriction sites of pCW-NPR, a human NADPH-cytochrome P450 reductase(NPR)-containing bicistronic expression vector (Parikh et al., 1997).

Bacterial expression

Bacterial harvest and membrane preparation were performed as described previously (Gillam et al., 1993; Guengerich and Martin, 2006). *E. coli* DH5α cells were transformed with the expression constructs and grown overnight at 37°C in Luria - Bertani broth containing 50 μg/mL ampicillin. The overnight culture was inoculated 1:1000 into Terrific Broth medium containing 50 μg/mL ampicillin and 1 mM thiamine. Cultures were incubated at 37°C with shaking at 200 rpm until they attained an OD_{600} of 0.5 - 0.7, then were supplemented with 1 mM isopropyl-β-D-thiogalactopyranoside (Amresco, Solon, OH) and 0.5 mM δ-aminolevulinic acid (Cayman Chemical, Ann Arbor, MI), a heme precursor ,and cultured for 24 h at 29°C with shaking at 200 rpm.

Culture was then chilled on ice and centrifuged at 3,800 × *g* for 20 min. The cell pellets were washed with PBS, and the cells were weighed and resuspended in 100 mM Tris-acetate buffer, pH 7.6, containing 500 mM sucrose, and 0.5 mM ethylenediaminetetraacetic acid (EDTA). Lysozyme was added to 0.2 mg/mL, and the suspensions were diluted two-fold with distilled water before incubation on ice for 30 min. The resulting spheroplasts were sedimented at 3,800 × *g* at 4°C for 20 min, and resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (*v*/*v*), and 10 mM β-mercaptoethanol (ME).

Suspensions of spheroplasts were sonicated four times for 20 s each, on ice, and centrifuged at 10,000 × *g* at 4°C for 20 min. Supernatants were centrifuged at 180,000 × *g* at 4°C for 75 min. Sedimented membrane fractions were resuspended in 100 mM potassium phosphate buffer, pH 7.6, containing 6 mM magnesium acetate, 20% glycerol (*v*/*v*), and 10 mM ME. The membrane preparation was stored at −70°C until use.

CYP and heme contents

CYP content was determined by reduced CO difference spectra (Omura and Sato, 1964). Sodium dithionite was added to reduce ferric CYPs. Ferrous-CO CYP complexes were generated by passing CO gas through solutions of the ferrous CYPs. The spectra were collected on a spectrophotometer at room temperature. Heme content was quantified using a pyridine hemochromogen assay and calculated from the difference in absorption between 557 and 575 nm (Schenkman and Jansson, 2006; Sinclair et al., 2001).

Immunoblots

Immunoblots were performed using a primary anti-CYP1A1 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and a secondary horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (GenDepot, Barker, TX). Blots were developed using a

chemiluminescence reagent kit. Protein concentrations were determined with a BCA protein assay kit (Pierce Biotechnology, Rockford, lL) using bovine serum albumin as a standard.

Site-directed mutagenesis

Site-directed mutagenesis was performed to generate sequence variants of CYPs using an EZchange site-directed mutagenesis kit (Enzynomics, Daejeon, South Korea) according to the manufacturer's instructions. Primers used for mutagenesis are listed in Table 1.

7-Ethoxyresorufin *O*-dealkylation (EROD)

7-ethoxyresorufin (ER) is a CYP1A1 probe substrate. EROD activities were assayed by fluorometric detection of resorufin using excitation and emission wavelengths of 544 and 595 nm, respectively (Chang and Waxman, 2006; Shimada and Yamazaki, 1998). The reaction mixture contained 20 μg of dilauroylphosphatidyl choline, 0.5 - 20 μM ER dissolved in dimethylsulfoxide, and *E. coli* membrane fractions in a total volume of 0.5 mL of 100 mM potassium phosphate buffer, pH 7.4. Following a 5 min pre-incubation at 37°C in a water bath, reactions were initiated by the addition of the NADPH-generation system (final concentrations 0.25 mM $NADP^+$, 2.5 mM glucose 6-phosphate, 0.25 IU of yeast glucose 6-phosphate dehydrogenase). Incubations were terminated after 10 min by the

addition of 1 mL ice-cold methanol.

Sequence alignment

To compare the amino acid sequences of CYP1A1s from various species, a multiple-sequence alignment was generated using the ESPript 3.0 alignment program (Robert and Gouet, 2014).

Statistics

All data are expressed as mean ± standard deviation (SD). Statistical analysis was performed with SPSS 19.0 for Windows (SPSS, Chicago, IL). Differences between sample groups were analyzed using the unpaired Student's *t*-test and one-way analysis of variance followed by the Tukey's *post hoc* test. A *P*-value of < 0.05 was considered significant.

Results

Sapsaree CYP1A1 cDNAs

A number of CYP1A1 cDNA sequences were aligned with Boxer cDNA sequences. Each cDNA contained an open reading frame of 1575 nucleotides, encoding 525 amino acids, including an initiation codon ATG and a termination codon TAG. Based on these alignments, two kinds of CYP1A1 cDNA sequences, Sap1 and Sap2, were found and registered to NCBI (Accession No. KP340900; KP340901). Both the Sap1 and the Sap2 variants have an amino acid change from a Ser codon to a Pro codon at position 522 (TCT to CCT). Additionally, the Sap2 variant differed from Sap1 variant by having an G → T transition at nucleotide 149, thus changing codon 50 from Trp to Leu (TGG to TTG) (Fig. 1).

Expression in *E. coli*

Whole *E. coli* cells expressing Sap1 variant and Sap2 variant as well as their membrane fractions were used for CO-difference spectra (Fig. 2). The cellular CYP content (*i.e.*, holoenzyme) in *E. coli* expressing the Sap2 variant was about 1.7-fold higher than that for the Sap1 variant ($P < 0.05$; Fig. 2A). The membrane fraction expressing the Sap2 variant also had about 1.8-fold higher CYP content than that of the Sap1 variant ($P < 0.05$; Fig. 2B). Based

upon immunoblots, the level of total CYP1A1 protein (apoprotein + holoprotein) in the whole cells and in membrane fractions of *E. coli* expressing two canine CYP1A1 variants was similar to the level in cells and membranes. These observations indicate that the expression of the apoprotein and its incorporation into *E. coli* membranes were not affected by the substitution. *E. coli* transformed with human CYP1A1 WT was used as a positive control. And *E. coli* transformed empty control vector (EV) was used as a negative control, for which CYP content and CYP1A1 protein expression were undetectable.

The heme content in the membrane fractions of *E. coli* expressing the Sap2 variant was 1.64-fold higher than that for the Sap1 variant and about 5-fold higher than in the EV control group ($P < 0.05$; Fig. 2C). The membrane fraction of *E. coli* transformed with EV had a heme level of 0.20 nmol/mg, which may represent endogenous heme levels in the *E. coli* membrane fraction. After deducing EV heme levels from those of Sap1 and Sap2 groups, the heme level of the Sap2 membrane fraction was 1.9-fold higher than that for the Sap1 variant [(0.64 - 0.20)/(1.05 - 0.20)=1.933)], which is similar to CYP level differences in whole *E. coli* cells and membrane fractions (Fig. 2A and 2B). These findings suggest that the Sap2 variant results in an increment of holo-CYP1A1 expression by increasing the heme content of the protein without affecting the expression of CYP1A1 apoprotein. We therefore hypothesize that the position of codon 50 within canine CYP1A1 is an important residue, which alters the ordered framework of the protein structure and

causes an increase of heme incorporation.

Enzyme activities of *E. coli*

Membrane fractions of *E. coli* expressing Sap1 variant and Sap2 variant were used for EROD enzyme assay. In terms of nmol/min/mg protein, EROD activities of the Sap1 variant-expressing membranes exhibited almost equivalent to those of the Sap2 variant-expressing membranes ($P < 0.05$; Fig. 3A). However, catalytic activities (measured in product formed per CYP content) of the Sap2 variant-expressing membranes were 60.3% of the Sap1 counterparts, respectively ($P < 0.05$; Fig. 3B).

Substitution of human CYP1A1

CYP content was measured in *E. coli* expressing the Trp46Leu variant and human CYP1A1 WT protein (Fig. 4A and 4B). The cellular CYP content in *E. coli* expressing the Trp46Leu variant was about 1.2-fold higher than that for the WT ($P < 0.05$; Fig. 4A). The membrane fraction expressing the Trp46Leu variant also had 1.2-fold higher CYP content than that of the WT ($P < 0.05$; Fig. 4B). The total CYP1A1 protein level of the variant was similar to that of WT in the whole cells and membrane fractions.

Membrane fractions of *E. coli* expressing Trp46Leu variant and WT proteins were used for EROD enzyme assay. EROD

activities (measured in product formed per mg protein) of the variant-expressing membranes were similar to those of the WT counterparts, respectively ($P < 0.05$; Figs. 4C). However, EROD activities for the variant-expressing membranes were 80% of those of the WT-expressing membranes in terms of nmol/min/nmol CYP, respectively ($P < 0.05$; Figs. 4D).

Substitution of codon 50

The CYP content was measured in *E. coli* expressing various single-residue variants of codon 50 (Fig. 5 and 7). The expression levels of total CYP1A1 protein were similar among the sample groups analyzed. *E. coli* expressing a small hydrophobic side chain such as Ala had CYP contents that were 80% of that for the Sap1 group ($P < 0.05$; Fig. 5). The CYP contents following substitution with residues having large hydrophobic side chains, Met and Phe, were almost equivalent of that for the Sap1 group. However, substitution to Val, having a hydrophobic side chain, resulted in 1.3-fold increase of the CYP content compared with the Sap1 variant. Additionally, *E. coli* expressing Ile-50 variant, which have similar residue masses to Leu, had CYP content that was higher by 1.6-fold than cells expressing the Sap1 variant and was similar to that of the Sap2 variant ($P < 0.05$; Fig. 5). In contrast, substitution to residues having polar or charged side chains such as Lys, Asp, and Asn caused >40% reduction of CYP content in *E. coli* expressing the Sap1 variant ($P < 0.05$; Fig. 7).

Catalytic activities of the *E. coli* membrane fractions expressing substitution of codon 50

Membrane fractions of *E. coli* expressing two canine CYP1A1 proteins and single-residue variants were used in the enzyme assay. In terms of nmol/min/mg protein, the substitution with hydrophobic amino acid residues did not affect EROD activities ($P < 0.05$; Fig. 6A). However, Val-50, Ile-50, and the Sap2 variant-expressing membranes showed lower EROD activities than those of the other variants in terms of nmol/min/nmol CYP ($P < 0.05$; Fig. 6B).

Compared to the Sap1 and Sap2 variants, the substitution with a polar or a charged amino acid residues resulted in a slight decrease in EROD activities (nmol/min/mg protein) ($P < 0.05$; Fig. 8A). In contrast, the variants having a polar or a charged residues showed similar EROD activities (measured in product formed per CYP content) to those of the Sap2 variant, ($P < 0.05$; Fig. 8B).

Discussion

The current study showed that the Sap2 variant, having leucine at position 50, resulted in an increase of holoenzyme expression compared to the Sap1 variant (Fig. 2). In contrast, the differences exhibited a marked difference in catalytic properties of the recombinant CYP1A1 enzymes of canine. We employed one substrate, ethoxyresorufin (ER), for comparison of the functions in two canine CYP1A1 enzymes. ER is a good substrate for CYP1A1. The EROD activities of the Sap2 variant-expressing membranes were much lower than those of the Sap1 variant-expressing membranes in terms of nmol/min/nmol CYP (Fig. 3B). This is the first study of canine CYP1A1 variants. These findings suggest that the substitution of codon 50 by leucine increased heme incorporation, whereas binding affinity for substrates decreased compared to the Sap1 variant.

The canine CYP1A1 amino acid sequence has a higher identity with human CYP1A1 (82%) than with rodent CYP1A1 (77%), and is 13 amino acid longer than the human orthologs. We also conducted site-directed mutagenesis in human CYP1A1 and showed the same results with canine CYP1A1 (Fig. 4).

Proper folding of apo-CYPs is required for heme insertion, and the heme plays an essential role as a template for final protein folding (Correia et al., 2011). The difference between two canine

CYP1A1 sequences was a single amino acid at position 50, which is predicted to be located in codon 46 of human CYP1A1. In human CYP1A1, the PR region has the sequence PPGPWGWPLIGH, where the seven residue is Trp46. In nearly every microsomal CYP molecule, a Pro-rich (PR) region is present following an *N*-terminal signal anchor sequence and a short hydrophilic linker sequence (Williams et al., 2000). The PR region has an important role in the proper folding of various CYP enzymes, especially prior to heme binding (Lee et al., 2015).

A molecular model of the PR region and adjacent structural elements shows that the PR region appears not to be directly involved in heme binding, but indirectly interacts with heme through β-sheet 1 and SRS-5 segments (Lee et al., 2015). Given the critical functions of the PR region's initial residues in holo-CYP1A1 formation, a Trp46Leu substitution in close proximity may have an influence on those residues' structural role in holo-CYP1A1 formation.

Arg77 appears to form a hydrogen bond with Trp46 and to be one of the key residues of β-strand 1-1 that is responsible for interactions with residues of the PR region (Fig. 9). The residue is likely to play an important role in the formation of holo-CYP1A1 enzyme (Lee et al., 2015). The potential change of interactions between Trp46 and Arg77, induced by Trp46Leu substitution, may affect holo-CYP1A1 enzyme formation.

To evaluate the effects of the substitution of amino acid

residue at position 50 in canine CYP1A1, site-directed mutagenesis was conducted. The level of holoenzyme formation on the substitution by Ile was equivalent to that of Sap2 variant having side chain of similar mass, Leu (Fig. 5). In contrast, substitution of codon 50 with residues having other hydrophobic chains resulted in similar levels of holoenzyme formation for the Sap1 variant and decreased levels for the Sap2 variant (Fig. 5). The substitution of residue with a polar and charged side chain exhibited decreased levels of CYP content compared to the Sap1 variant (Fig. 7). It is thus possible that the amino acid residue at position 50, which had Leu and Ile, is important for efficient anchoring of the canine CYP1A1 proteins in the endoplasmic reticulum membrane.

In the catalytic activities, *E. coli* membranes expressing hydrophobic amino acid residues had similar activities of EROD in terms of nmol/min/mg protein (Fig. 6A). On the other hand, the substitution of codon 50 with residues, such as Ile and Val, had lower levels of enzyme activities (nmol/min/nmol CYP) than those of the Sap1 variant-expressing membranes (Fig. 6B). In case of *E. coli* membranes having polar and charged amino acid residues, the levels of the enzyme activities (measured in product formed per CYP content) much lower than those of two canine CYP1A1 variants-expressing membranes (Fig. 8). Based upon these findings, we suggest that residues having Val, Ile, and Leu alter the ordered framework of the protein, affect interaction with substrate recognition site, and lead to a decrease of binding affinity for substrates.

Taken together, these findings suggest that codon 50 in canine CYP1A1 substitution leads to a structural disturbance of CYP1A1, affecting its holoenzyme formation and catalytic activities. It is proposed that the variant having Val, Ile and Leu may have reduced CYP1A1 activity, compared with the Sap1 variant, in various canine tissues and that an individual carrying the variant allele may have different susceptibility to adverse health effects of environmental chemicals that are metabolized via CYP1A1-dependent pathways. Further studies are warranted to extend our understanding of the importance of the polymorphism in environmental health, especially with respect to the influence of the polymorphism on gene expression and regulation, as well as to the association between the polymorphism and the incidence of environmentally associated diseases.

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Figures

Fig. 1

Sequence comparison of CYP1A1s from various animal species. The different species and associated accession numbers for CYP1A1s are as follows; *C. lupus* (XP_003433938.1), *H. sapiens* (NP_000490.1), *P. troglodytes* (XP_001137654.1), *B. taurus* (XP_005222075.1), *M. mulatta* (NP_001035328.1), *M. musculus* (NP_001129531.1), and *R. norvegicus* (NP_036672.2). Conserved residues are in blue boxes; identical residues are shown with a red background, similar residues in red typeface. Different residue between two canine CYP1A1 variants (codon 50) are indicated by a black triangle.

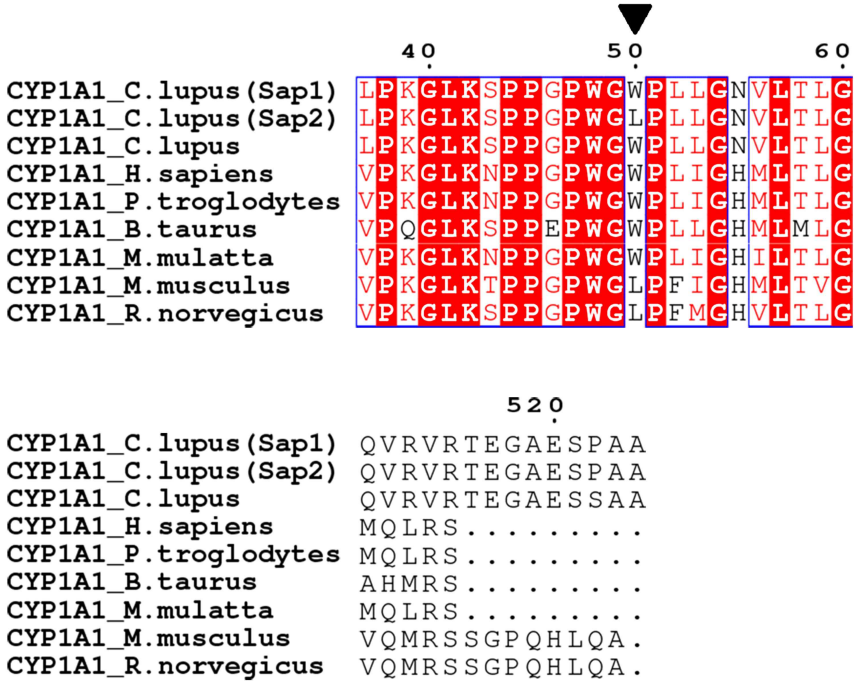

40
50
60
CYP1A1_C.lupus(Sap1) LPKGLKSPPGPWGWPLLGNVLTLG
CYP1A1_C.lupus(Sap2) LPKGLKSPPGPWGLPLLGNVLTLG
CYP1A1_C.lupus LPKGLKSPPGPWGWPLLGNVLTLG
CYP1A1_H.sapiens VPKGLKNPPGPWGWPLIGHMLTLG
CYP1A1_P.troglodytes VPKGLKNPPGPWGWPLIGHMLTLG
CYP1A1_B.taurus VPQGLKSPPEPWGWPLLGHMLMLG
CYP1A1_M.mulatta VPKGLKNPPGPWGWPLIGHILTLG
CYP1A1_M.musculus VPKGLKTPPGPWGLPFIGHMLTVG
CYP1A1_R.norvegicus VPKGLKSPPGPWGLPFMGHVLTLG
520
CYP1A1_C.lupus(Sap1) QVRVRTEGAESPAA
CYP1A1_C.lupus(Sap2) QVRVRTEGAESPAA
CYP1A1_C.lupus QVRVRTEGAESSAA
CYP1A1_H.sapiens MQLRS.....
CYP1A1_P.troglodytes MQLRS.....
CYP1A1_B.taurus AHMRS.....
CYP1A1_M.mulatta MQLRS.....
CYP1A1_M.musculus VQMRSSGPQHLQA.
CYP1A1_R.norvegicus VQMRSSGPQHLQA.

Fig. 2

Expression of two canine CYP1A1 variants. *E. coli* cells were transformed either with pCW-NPR vector including two canine CYP1A1 variants or human CYP1A1 WT cDNAs (Hm), or with EV. Total CYP contents were quantified using reduced CO-difference spectra in (A) whole cells and (B) the membrane fractions. Lower insets represent total CYP1A1 protein expression in whole cells and membrane fractions, which were assessed by immunoblotting. (C) Heme contents were determined in the membrane fractions. Each bar represents the mean ± SD of six independent samples. Different lower-case letters indicate significant differences in each panel ($P < 0.05$, Tukey's *post hoc* test).

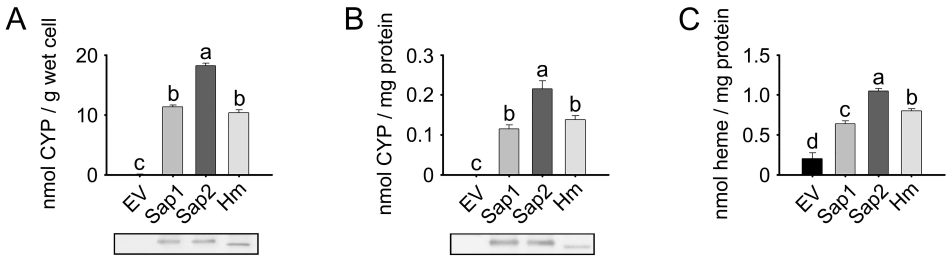

A
nmol CYP / g wet cell
20
10
0
c
b
a
b
EV
Sap1
Sap2
Hm
B
nmol CYP / mg protein
0.3
0.2
0.1
0
c
b
a
b
EV
Sap1
Sap2
Hm
C
nmol heme / mg protein
1.5
1.0
0.5
0
d
c
a
b
EV
Sap1
Sap2
Hm

Fig. 3

Catalytic activities of two canine CYP1A1 variants. (A) EROD activities (measured in product formed per mg protein) of *E. coli* membrane fractions expressing two canine CYP1A1 variants and human CYP1A1 WT. (B) EROD activities (measured in product formed per CYP content) in *E. coli* membrane fractions expressing two canine CYP1A1 variants and human CYP1A1 WT. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of six independent samples ($P < 0.05$, Tukey's *post hoc* test).

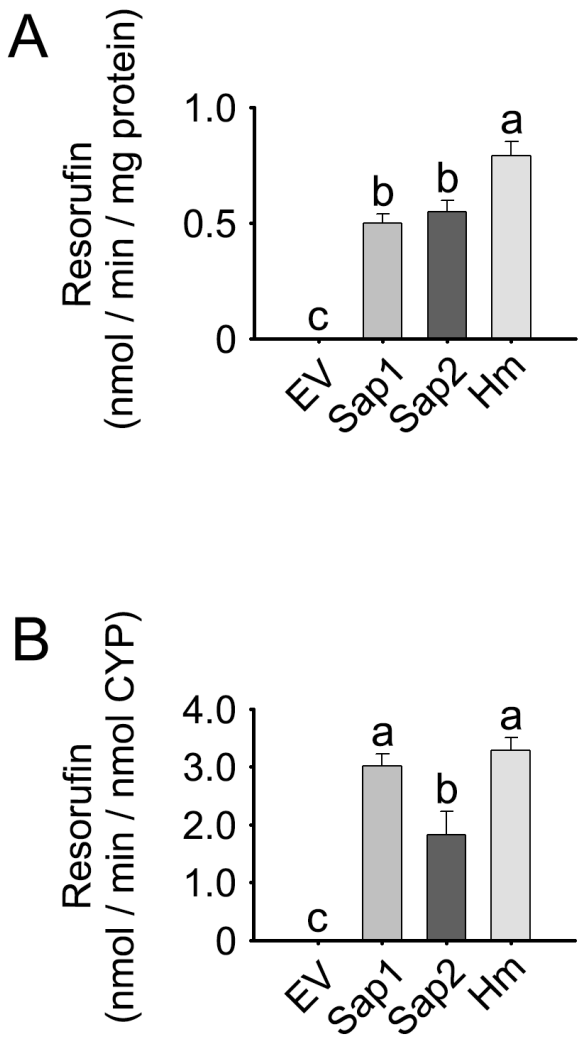

A
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
c
b
b
a
EV
Sap1
Sap2
Hm
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
a
EV
Sap1
Sap2
Hm

Fig. 4

CYP contents and CYP1A1 activities in *E. coli* expressing human CYP1A1 WT and its Trp46Leu variant. CYP contents in (A) whole cells and (B) the membrane fractions. (C) EROD activities (measured in product formed per mg protein) of *E. coli* membrane fractions expressing the variant and WT proteins. (D) EROD activities (measured in product formed per CYP content) of *E. coli* membrane fractions expressing the variant and WT proteins. Detection of total CYP1A1 protein was performed with immunoblots of whole cells and membrane fractions of *E. coli*. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

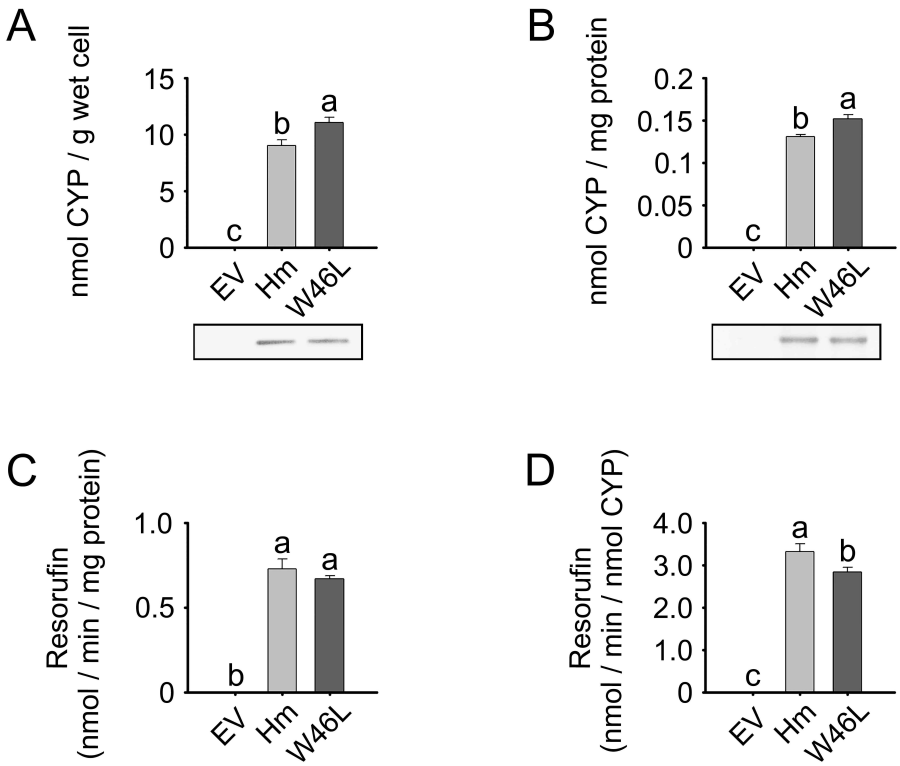

A
nmol CYP / g wet cell
15
10
5
0
c
b
a
EV
Hm
W46L
B
nmol CYP / mg protein
0.2
0.15
0.1
0.05
0
c
b
a
EV
Hm
W46L
C
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
b
a
a
EV
Hm
W46L
D
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
EV
Hm
W46L

Fig. 5

CYP contents for two canine CYP1A1 variants and single-residue variants, having hydrophobic amino acid of codon 50. Two canine CYP1A1 proteins and hydrophobic amino acid residue variants are arranged in order of small to large masses of substituting residues in (A) whole cells and (B) the membrane fractions. Total CYP1A1 protein expression was analyzed with immunoblots. Each bar represents the mean ± SD of four independent samples. Different lower-case letters above the bars indicate a significant difference among the groups ($P < 0.05$, Tukey's *post hoc* test).

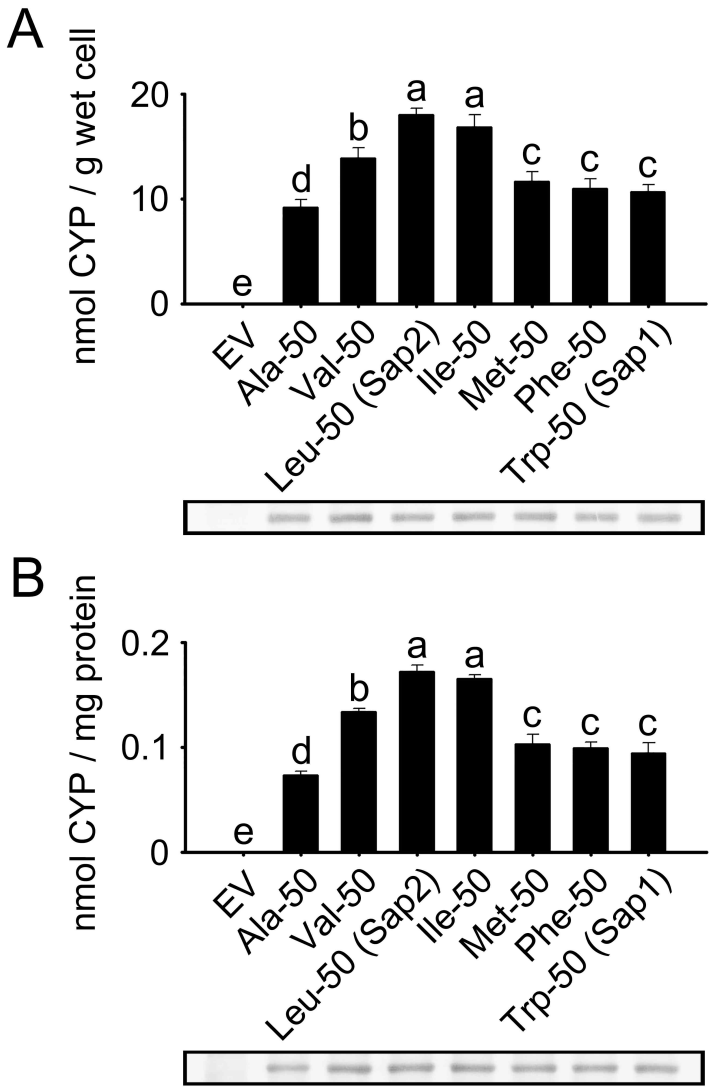

A
nmol CYP / g wet cell
20
10
0
e
d
b
a
a
c
c
c
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)
B
nmol CYP / mg protein
0.2
0.1
0
e
d
b
a
a
c
c
c
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)

Fig. 6

Enzyme activities in *E. coli* membrane fractions expressing two canine CYP1A1 variants and hydrophobic amino acid residue variants of codon 50. (A, B) EROD activities of *E. coli* membrane fractions. A, nmol/min/mg protein; B, nmol/min/nmol CYP. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

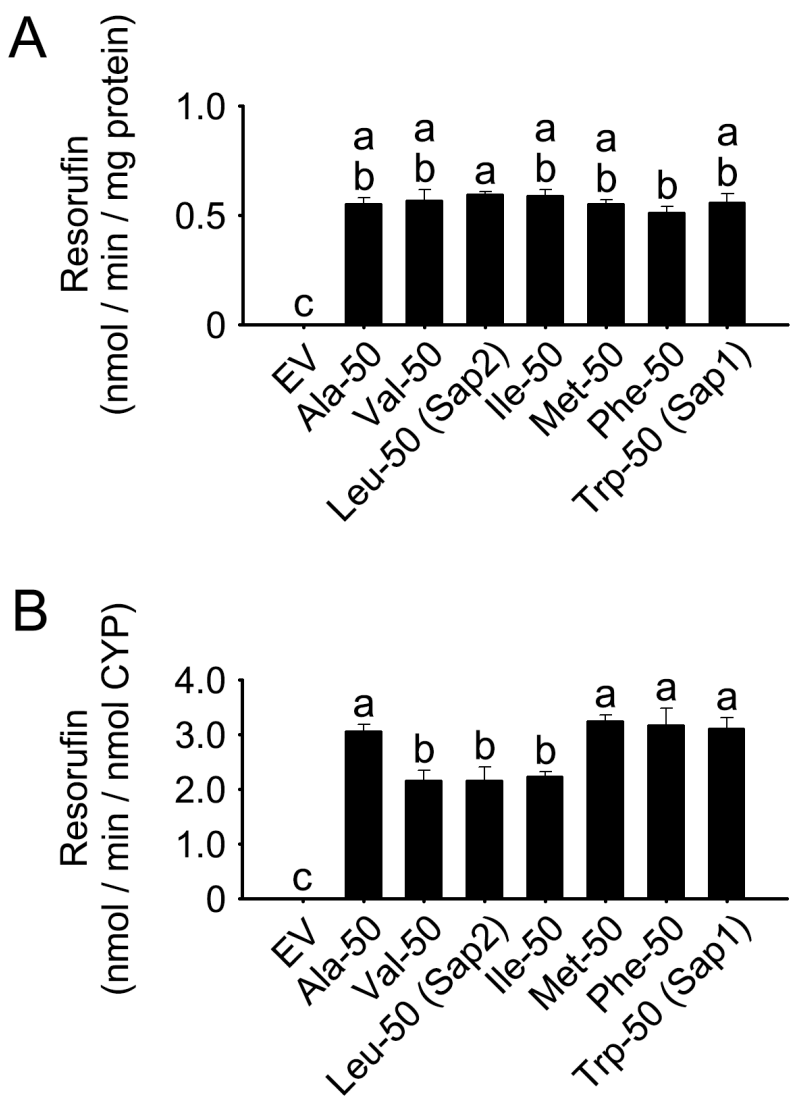

A
Resorufin
(nmol / min / mg protein)
1.0
0.5
0
c
a
b
a
b
a
a
b
a
b
b
a
b
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
c
a
b
b
b
a
a
a
EV
Ala-50
Val-50
Leu-50 (Sap2)
Ile-50
Met-50
Phe-50
Trp-50 (Sap1)

Fig. 7

CYP contents in *E. coli* expressing polar or charged amino acid residue variants of codon 50. (A) Whole cells and (B) the membrane fractions. *E. coli* cells were transformed either with pCW-NPR vector harboring cDNAs or with EV. Total CYP1A1 protein expression was analyzed with immunoblots. Each bar represents the mean ± SD of four independent samples. Different lower-case letters above the bars indicate a significant difference among the groups ($P < 0.05$, Tukey's *post hoc* test).

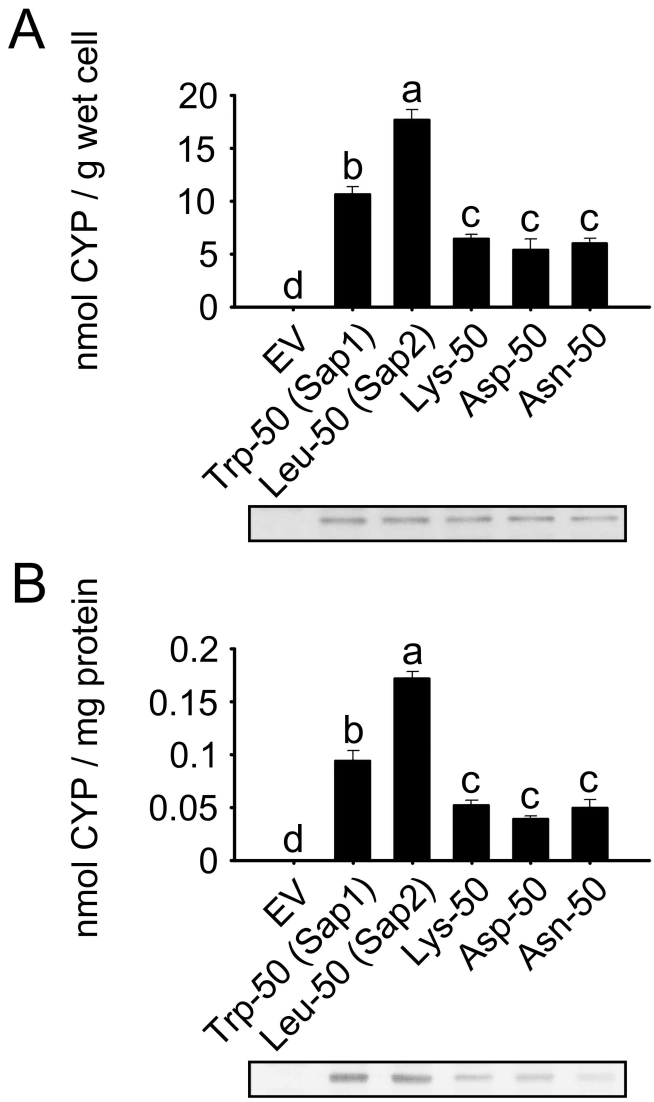

A
nmol CYP / g wet cell
20
15
10
5
0
a
b
c
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50
B
nmol CYP / mg protein
0.2
0.15
0.1
0.05
0
a
b
c
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50

Fig. 8

Catalytic activities in *E. coli* membrane fractions expressing two canine CYP1A1 variants and polar or charged amino acid residue variants of codon 50. (A, B) EROD activities of *E. coli* membrane fractions. A, nmol/min/mg protein; B, nmol/min/nmol CYP. Detection of CYP1A1 protein was performed with immunoblots. Each bar represents the mean ± SD of four independent samples ($P < 0.05$, Tukey's *post hoc* test).

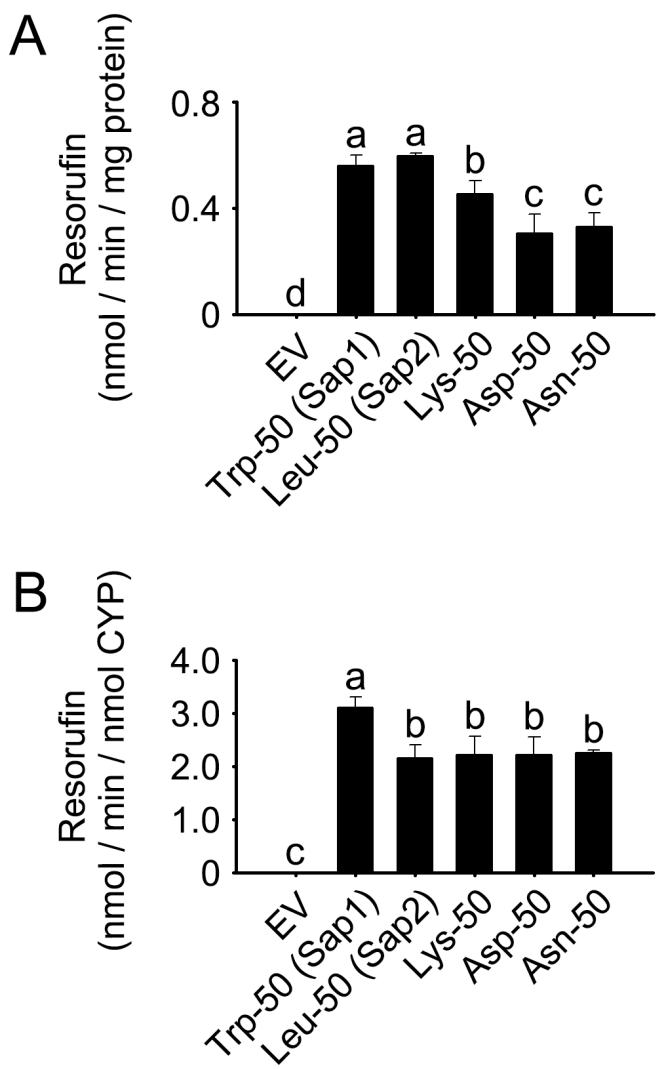
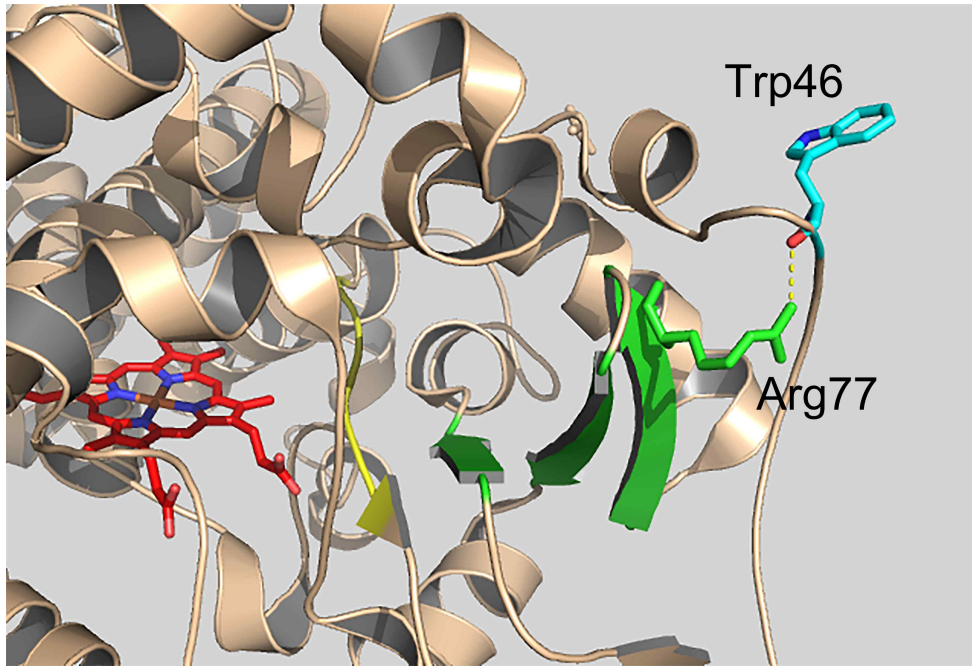

A
Resorufin
(nmol / min / mg protein)
0.8
0.4
0
a
a
b
c
c
d
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50
B
Resorufin
(nmol / min / nmol CYP)
4.0
3.0
2.0
1.0
0
a
b
b
b
b
c
EV
Trp-50 (Sap1)
Leu-50 (Sap2)
Lys-50
Asp-50
Asn-50

Fig. 9

A three-dimensional structure of CYP1A1 (PDB ID: 4I8 V). A ribbon model of the PR region harboring Trp46 and adjacent structural segments such as β-sheet 1, β-strand 2-2, SRS-5, and heme.


Trp46
Arg77

Table

Table 1. Oligonucleotide primer sequences used for cDNA cloning, *N*-terminal modification, and site-directed mutagenesis. [a]Coding sequences corresponding to the mutated amino acids are underlined and in bold.

	Primer	Oligonucleotide sequence (5' to 3')[a]	Description
	CYP1A1-F	CAG CTG TCT TGA GGT CTC TAC GC	Forward primer for cDNA cloning of canine CYP1A1
	CYP1A1-R	ACC CAG ACA GGC CAG GTA GAC AG	Reverse primer for cDNA cloning of canine CYP1A1
	N-ter-F	CG CTG GTG CAT ATG GCT TCT ATG TTT AGA CTT TCT ATT CCC	Forward primer for *N*-terminal modification of canine CYP1A1
	N-ter-R	GCT CTA GAC TAG GCT GCA GGG CTC	Reverse primer for *N*-terminal modification of canine CYP1A1
	Ala50-F	G GGC **GCT** CCC CTG CTC GGG AAC	Forward primer for site-directed mutagenesis of coding sequence of codon 50 into a Ala
	Ala50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site-directed mutagenesis of coding sequence of codon 50 into a Ala

Canine CYP1A1	Phe50-F	G GGC **TTT** CCC CTG CTC GGG AAC	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Phe
	Phe50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Phe
	Val50-F	G GGC **GTG** CCC CTG CTC GGG AAC	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Val
	Val50-R	CAG GGC CCC GGT GGA CTC TTC AG	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Val
	Met50-F	GC **ATG** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Met
	Met50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Met
	Ile50-F	GC **ATT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Ile
	Ile50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Ile
	Lys50-F	GC **AAG** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Lys
	Lys50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Lys
	Asp50-F	GC **GAT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Asp
	Asp50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Asp

	Asn50-F	GC **AAT** CCC CTG CTC GGG AAC GTG	Forward primer for site—directed mutagenesis of coding sequence of codon 50 into a Asn
	Asn50-R	CCC AGG GCC CCG GTG GAC TCT TC	Reverse primer for site—directed mutagenesis of coding sequence of codon 50 into a Asn
Human CYP1A1	W46L-F	C **TTA** CCT CTG ATT GGG CAC ATG	Forward primer for site—directed mutagenesis of coding sequence of Trp46 into a Leu
	W46L-R	CCC CAT GGC CCT GGT GGA TTC	Reverse primer for site—directed mutagenesis of coding sequence of Trp46 into a Leu

국문 초록

재조합 발현을 이용한 개 cytochrome P450 1A1 변이형의 생화학적 분석

Cytochrome P450 (CYP)는 heme-thiolate 효소로서 대부분의 생물 종에서 발견되며, 포유동물에서 약물, 생체 내 물질(endogenous substances), 그리고 생체 외 물질(xenobiotics)의 최초대사와 관련 되어 있다. CYP는 주로 세포 내 소포체(endoplasmic reticulum)에 존재한다. CYP1A1은 간 외 조직, 주로 폐 조직에서 매우 높게 발현되며, 환경 발암물질(environmental carcinogen)과 환경 호르몬의 대사에 매우 중요한 역할을 담당하고 있다. 인간 CYP1A1 유전자는 다형성을 나타내며, 소수의 단일 염기서열다형성이 보고 및 연구 되었다. 하지만 개 CYP1A1 유전자의 다형성에 대한 연구는 아직 보고된 바 없다.

본 연구에서는 삽살개의 cDNA 서열을 분석하여 2종류의 CYP1A1 변이형을 발견하였고, 이를 각각 Sap1과 Sap2라고 명명하였다. 2종류의 CYP1A1 변이형은 50번째 아미노산 서열에서 차이를 보였다. Sap1은 50번째 아미노산에 Trp이 위치하였고, Sap2는 Leu이 위치하였다. 2종류의 CYP1A1 변이형은 대장균 세포 내에서의 CYP1A1 아포효소(apoenzyme)의 전사, 번역 효율에 영향을 주지 않았으며, 대장균 세포막에 CYP1A1 단백질이 결합되는 정도에도 영향을 주지 않았다. 그러나 Sap1의 CYP 완전효소(holoenzyme)의 양은 Sap2에 비해 낮은 것으로 나타났는데, 이것은 Sap1 변이형 단백질이 heme기가 결여된 무기능 단

백질 형태로 존재한다는 것을 시사한다. 하지만, 제 기능이 유지된 완전 효소 양을 기준으로 하여 Sap1과 Sap2 단백질의 7-에톡시레소루핀-*O*-디에틸라아제(EROD) 활성을 비교할 경우, Sap2의 활성이 Sap1의 활성에 비해 낮았다. 이 결과를 토대로, 50번째 아미노산의 변이는 단백질 구조를 변화시키고 heme기의 결합에 영향을 미친다는 것이 예상 되었다.

Site-directed mutagenesis 기술은 CYP1A1의 구조와 기능의 상관관계를 규명하고 중요한 아미노산 서열을 알아내는데 매우 유용하게 사용되었다. CYP1A1의 구조 내 상호작용의 특징을 설명하기 위해 50번째 아미노산을 다른 아미노산으로 치환하여 동일한 실험을 수행하였다. 그 결과 50번째 아미노산 위치에 잔기의 분자량이 40-60 정도 되는 소수성 아미노산인 Val, Leu, Ile이 위치하는 경우 CYP 단백질의 heme기 결합 능력이 증가하는 양상을 보였다. 반면 7-에톡시레소루핀-*O*-디에틸라아제 활성은 감소하는 양상을 보였다.

이러한 결과는 50번째 아미노산의 변이가 CYP 단백질의 heme기의 유입(heme incorporation)과 효소 활성에 영향을 줌으로써, 기능 변화를 유발한다는 것을 시사한다.

주요어: Cytochrome P450 1A1 (CYP1A1), Canine CYP1A1, heme기, 완전효소 (holoenzyme), 에톡시레소루핀-*O*-디에틸라아제 (EROD)

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