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Expression and Characterization of Dog CYP2D15 Using Baculovirus Expression System[1]

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Dog CYP2D15 was expressed in Sf9 cells with a recombinant baculovirus. Infection of Sf9 insect cells with a recombinant dog CYP2D15-virus resulted in the expression of a protein which cross-reacted with a polyclonal antibody against a dog CYP2D15-specific peptide. The difference spectrum of CO-complex of reduced P450 of the infected cell microsomes had a maximal absorbance at 449 nm. The specific content of P450 was calculated to be 0.56 nmol/mg of Sf9 cell microsomal protein. Although the expressed dog CYP2D15 showed high catalytic activity for the hydroxylations of bunitrolol and imipramine at low substrate concentration (10 μM), the catalytic activity for that of debrisoquine (50 μM) was extremely low as compared with that of CYP2D from other species. Dog liver microsomes also showed bunitrolol and imipramine hydroxylase activities, but not debrisoquine hydroxylase activity at the same substrate concentrations. In addition, the expressed CYP2D showed high catalytic activity for imipramine *N*-demethylation. Thus, our study reveals that the expressed dog CYP2D15 engages in high catalytic activity and has a unique substrate specificity from other CYP2D subfamilies. Western blot analysis suggested that the dog CYP2D15 contents were less than 4% of the total liver P450 content, assuming that 100% of expressed CYP2D15 incorporated heme.

Key words: baculovirus expression, CYP2D, cytochrome P450, dog, drug metabolism.

P450 enzymes are important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous and exogenous compounds. The P450 superfamily consists of more than 500 genes, which are classified according to the currently available amino acid sequence data into 74 families (*1*).

The P450 2D (CYP2D) subfamily plays an important role in the metabolism of over 30 clinically used drugs (*2*). Intense studies have been carried out on the genetic polymorphism of CYP2D with regard to individual ability to metabolize debrisoquine/sparteine (*3-7*). Recombinant human CYP2D6 has recently been expressed in several heterologous expression systems including yeast (*8, 9*), bacteria (*10, 11*), insect cells (*12, 13*), and mammalian cells (*14*).

Since dogs are commonly used as experimental animals in pharmacokinetic and metabolic studies for the development of new drugs, it is important to understand the properties of the CYP2D subfamily in dogs. Although the purification of P450 is extremely useful as a means to investigate unknown P450s, the examination of the catalytic properties of purified CYP2D is hampered by the difficulty in the preparation of CYP2D from liver microsomes. Although Sakamoto *et al.* (*15*) have reported the expression of dog CYP2D15 in COS-7 cells, they were not able to determine the rate of drug metabolism as CYP2D specific activity owing to the low yield of CYP2D expressed. In this study, we found that the baculovirus expression system afforded the high-level expression of recombinant dog CYP2D protein, providing a sufficient amount for studies of drug metabolism.

In summary, we cloned CYP2D subfamily cDNA from dog liver and expressed recombinant dog CYP2D using the baculovirus expression system. We characterized the expressed CYP2D15 by measuring its ability to metabolize several drugs. In addition, we quantified the specific content of CYP2D15 in dog liver microsomes by using an anti-CYP2D15 peptide antibody.

[1] The nucleotide sequence data reported in this paper have been submitted to the DDBJ, EMBL, and GenBank nucleotide sequence databases with the accession number AB004268. The nomenclature number CYP2D15 was provided by Nelson *et al.* (*1*). This work was supported in part by Grants-in-Aid from the Ministry of Education, Science, Sports and Culture of Japan to S.F. (No. 07558236), A.K. (No. 08456155), and H.I. (No. 09306021).

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MATERIALS AND METHODS

Chemicals and Reagents—Bunitrolol and 4-hydroxybunitrolol were gifts from Boehringer Ingelheim (Osaka). Imipramine hydrochloride, *N*-demethylimipramine hydrochloride, and nortriptyline hydrochloride were obtained from Sigma Chemical (St. Louis, MO). 2-Hydroxyimipramine was a gift from Geigy (Basel, Switzerland). Debrisoquine sulfate and 4-OH-debrisoquine hydrochloride were gifts from Roche Japan (Tokyo). NADPH, $NADP^+$, glucose-6-phosphate (G-6-P), and G-6-P dehydrogenase were purchased from the Oriental Yeast (Tokyo). All other chemicals are of analytical grade. Enzymes were from Takara (Otsu), Toyobo (Osaka), and New England Biolabs (Beverly, MA). [α-^{32}P]dCTP was purchased from Amersham Life Science (Tokyo). Nylon membrane was purchased from Schleicher & Schuell (Keene, NH). A λgt10 cDNA library from an adult male beagle dog liver was purchased from Clontech (Palo Alto, CA). A site-directed mutagenesis system (Mutan-G) was purchased from Takara. The mutagenic oligonucleotide primer, 5′-gtcagcagcccatagttagatctgtgcccgctgggctc-3′, was purchased from Saiensu Tanaka (Sapporo). A baculovirus expression system was purchased from Pharmingen (San Diego, CA). Reagents for cell culture were purchased from GIBCO BRL (Gaithersburg, MD).

Cloning and Sequencing of the Dog CYP2D cDNA—The cDNA library (3×10^5 plaques) was screened by using CYP2D6 cDNA (7). The probe was labeled with [α-^{32}P]-dCTP, using a random prime labeling kit (Nippon Genetics, Tokyo). The first screening was performed under nonstringent conditions. The condition for plaque hybridization was 60°C for 12 h in 10×Denhardt's and buffer A (0.05 M Tris-HCl, pH 8.0, 1 M NaCl, 10 mM EDTA, 0.1% SDS) containing 100 μg/ml heat-denatured salmon sperm DNA. The hybridized nylon membranes were washed twice at 60°C in buffer B (3×SSC, 0.1% SDS), then autoradiographed for 12 h at −80°C. Four positive clones (CFL-33, 38, 44, 93) were obtained. The insert cDNA was subcloned into the vector pUC18 at the *Eco*RI site, and the nucleotide sequences of the clone were determined from both ends. Analysis of the DNA sequences showed that these clones did not contain the full coding region, but were more than 75% identical to human CYP2D6. To obtain a full-length clone of the CYP2D subfamily in dog liver, a second screening of the same library (10^6 plaques) was performed, using the cDNA obtained in the first screening as a probe. Hybridization and washing were performed under stringent conditions at 65°C. The other conditions were the same as those fixed for the first screenings. The cDNAs with the largest inserts (about 1.6 kbp, CFL-114) were subcloned into the vector pUC18 or M13mp18 at the *Eco*RI site and subjected to sequencing with an Applied Biosystems Model 373A DNA sequencer using fluorescent dye-terminators.

Construction of Recombinant Baculovirus and Protein Expression—The cDNA was subcloned into the pVL1392 transfer vector. The CFL-114 cDNA was inserted between the *Bgl*II and the *Eco*RI sites in the multiple cloning site of pVL1392. For maximum expression, the 5′ and 3′ noncoding regions of CFL-114 were removed. The 5′ nontranslated region was altered by using site-directed mutagenesis to create a *Bgl*II restriction site (plasmid; pCFL-mu114). At the same time, we also modified the translational initiation codon (AUG) context. Since an A at −3 (relative to AUG at +1, +2, +3) may be important in baculovirus expression systems (*16*), we changed G to A at the −3 position of the AUG context sequence. Mutagenesis was performed according to the method of Kramer and Fritz (*17*), following the instructions provided by the kit (Mutan-G) supplier. The mutated fragment was cut from pCFL-mu114 using *Dde*I, after which a T4 DNA polymerase was used to fill in to create the 1.55 kbp blunt-ended fragment. pUC18 vector was cut by *Sac*I, after which the *Sac*I sites were filled in and dephosphated to obtain the pUC18sac. The 1.55 kbp blunt-ended fragment from pCFL-mu114 and the pUC18sac were linked to obtain pMu-114. The 1.51 kbp *Bgl*II-*Eco*RI fragment from pMu-114 was then cloned into the *Bgl*II and *Eco*RI sites of pVL1392. The recombinant baculovirus was constructed with an expression kit (BaculoGold™ transfection kit) using Sf9 (*Spodoptera frugiperda*) cells. The purified pMu-114/pVL1392 (5 μg) and the BaculoGold™ DNA (0.5 μg) were cotransfected into Sf9 cells by calcium phosphate precipitation according to the manufacturer's protocol. After 4 days, the supernatant of the cotransfection plate was transfected into Sf9 cells. Recombinant protein was identified from SDS solubilized cell pellets 3 days after transfection, using Western blot analysis. Successive plaque purifications were performed and recombinant viral stocks were prepared in Sf9 cells. To measure the P450 reduced CO-binding absorption spectra and the catalytic activity, we infected Sf9 monolayer cells with the purified recombinant virus stocks at a multiplicity of infection (MOI) of 10 plaque-forming units per cell and added a hemin solution (1 mg/ml in 0.1 M ammonium hydroxide) to the media to give a final concentration of 2 μg/ml 24 h after infection. The cells were harvested 48 h after the addition of hemin, washed in PBS, and lysed by sonication and homogenization in lysate buffer (0.1 M sodium phosphate buffer, pH 7.4, containing 20% glycerol, 1 mM dithiothreitol, and 1 mM EDTA) and 1 mM phenylmethylsulfonyl fluoride. The cell lysates were centrifuged ($9{,}000\times g$) for 20 min to remove pelleted cells, and a membrane fraction was prepared by high speed centrifugation ($105{,}000\times g$) for 60 min. The membrane pellet was then resuspended in lysate buffer, and stored at −80°C.

Animals and Preparation of Liver Microsomes—Male beagle dogs (1.8 years old) were gifts from the Research Laboratories of New Drug Development (Iwamizawa). After dogs had been anesthetized with an intravenous injection of Nembutal, the carotid artery was excised to drain the blood. Dog livers were perfused *in situ* through the portal vein with ice-cold 1.15% KCl, then removed and diced. The diced livers were first minced in a mixer (HM-7SA, Nippon Rikagaku Kikai, Tokyo) at 1,000 rpm for 2 min then homogenized with 3 volumes of 1.15% KCl. Microsomes from the dog livers were prepared according to the method of Omura and Sato (*18*). The microsomal preparation was frozen with liquid nitrogen and stored at −80°C until use.

Assays of Drug-Metabolizing Activities of the Expressed CYP2D15 and Liver Microsomes—The reaction mixture contained 100 mM phosphate buffer, pH 7.4 (bunitrolol and debrisoquine metabolism) or 0.15 M Tris, pH 7.4 (imipramine metabolism), 3 mM (bunitrolol metabolism) or 10 mM (imipramine metabolism) or 20 mM (debrisoquine

metabolism) $MgCl_2$, 10 mM glucose-6-phosphate, substrate and liver microsomes or both the desired amount of microsomal membrane from Sf9 insect cells and NADPH P450 reductase. After preincubation at 37°C for 5 min, the reaction was initiated with NADPH (500 μM) and glucose-6-phosphate dehydrogenase (2 units). The addition of purified rat liver NADPH P450 oxidoreductase to the assay system was carried out by preincubating the proteins with the microsomal membrane from Sf9 insect cells at room temperature for 10 min.

The activity of bunitrolol 4-hydroxylase was determined by an HPLC procedure according to the method of Suzuki *et al.* (*19*) with slight modifications. The substrate concentration was 10 μM. The reaction mixture (1 ml) contained 20 pmol of P450 in insect cell microsomal membrane and 2.67 units of rat NADPH P450 oxidoreductase or 0.1 mg of dog liver microsomes.

Imipramine 2-hydroxylase and *N*-demethylase activities were determined by an HPLC procedure. The reaction was performed at 37°C in a shaking reactor for 2 min (the expressed CYP2D15) or 45 s (liver microsomes) in a 1 ml reaction mixture (30 pmol of P450 of insect cell microsomal membrane, 4 units of rat NADPH P450 oxidoreductase or 1 mg of dog liver microsomes). The substrate concentration was 10 μM. The reaction was terminated by the addition of 1 M sodium carbonate buffer (1 ml, pH 10). The metabolites were extracted from the reaction mixture with ethyl acetate (4 ml) after the addition of nortriptyline (2 nmol) as an internal standard. H_2SO_4 (0.2 ml, 0.01 N) was added to the ethyl acetate and mixed well for extraction of basic compounds into the acidic layer followed by centrifugation at 1,200×*g* for 10 min. The aqueous phase containing 2-OH and *N*-demethylimipramine was evaporated under reduced pressure after the addition of NaOH (0.2 ml, 0.01 N) to neutralize it. The resulting residue was dissolved in the HPLC mobile phase; H_2O:CH_3CN:CH_3OH:di-*n*-butylamine (48:53:50:2) and subjected to reverse-phase HPLC. The column (Inertsil ODS) was eluted at a flow rate of 1.8 ml/min. 2-OH imipramine, *N*-demethylimipramine, and nortriptyline were detected by an HPLC UV spectrophotometer (UV-970, Japan Spectroscopic, Tokyo) at 254 nm.

Activity of debrisoquine 4-hydroxylase was determined by an HPLC procedure according to the method of Suzuki *et al.* (*19*), with slight modifications. The reaction (0.5 ml) mixture contained 20 pmol of P450 in insect cell microsomal membrane and 2.67 units of rat NADPH P450 oxidoreductase or 0.5 mg of dog liver microsomes. The substrate concentration was 50 or 2,000 μM.

Quinine Inhibition Studies—To study the inhibitory effect of quinine on drug-metabolizing enzyme activities, quinine was added to the reaction mixture at various concentrations (1, 5, 10, and 100 μM). Other experimental conditions were the same as those described above, with the exception of the HPLC conditions for the imipramine assay [flow rate 1.0 ml/min, mobile phase H_2O:CH_3CN:CH_3OH:di-*n*-butylamine (55:25:5:1)]; this was modified to obtain the optimal retention times of quinine and imipramine metabolites for the best separation.

Other Methods—SDS-PAGE was carried out by the method of Laemmli (*20*). Spectra of P450 were measured as described by Omura and Sato (*21*). Protein concentrations were determined by the method of Lowry *et al.* (*22*). NADPH-P450 oxidoreductase was purified from phenobarbital-treated SD rats by the method of Ardies *et al.* (*23*). The reduction of cytochrome *c* by NADPH-P450 oxidoreductase was carried out as previously described (*24*). One unit of reductase activity is defined as the amount of enzyme that can reduce 1 μmol of cytochrome *c* per min. Western blotting was performed by the method of Winston *et al.* (*25*), using the dog CYP2D-specific anti-peptide IgG (*26*). Immunoreactive proteins were visualized with diaminobenzidine as the substrate. The peak area of the immunoreactive bands was quantified with NIH-Image (*27*).

RESULTS AND DISCUSSION

Cloning and Sequencing of CYP2D cDNA from Dog Liver—We isolated a cDNA from a dog liver cDNA library using a human CYP2D6 cDNA as a probe. Figure 1 shows the sequencing strategy. Our cloned cDNA was identical to the CYP2D15 that has already been reported (*15*), and, like Sakamoto *et al.*, we were unable to find more than one dog CYP2D subfamily, although we carried out non stringent screening with human CYP2D6 cDNA as a probe. Such results may suggest that there is predominantly or only one CYP2D form in male dog liver. Recently, several mammalians CYP2D subfamilies have been isolated (*7, 28-32*) [Lawton *et al.* (The Gene Bank, Accession No. U38218)]. Dog CYP2D was moderately similar in deduced amino acid sequence to the bovine, human, and monkey CYP2D subfamilies (74.8-76.4%), while showing lower similarities to the rat, mouse, and guinea pig CYP2D subfamilies (60.6-70.2%).

Expression of the Recombinant Dog CYP2D15—The recombinant dog CYP2D15 protein was expressed in Sf9 cells with a recombinant baculovirus. To compare this with the native protein in dog liver microsomes, we carried out Western blot analysis with anti-peptide antibody (*26*). Comigrated single bands appeared in dog liver microsomes

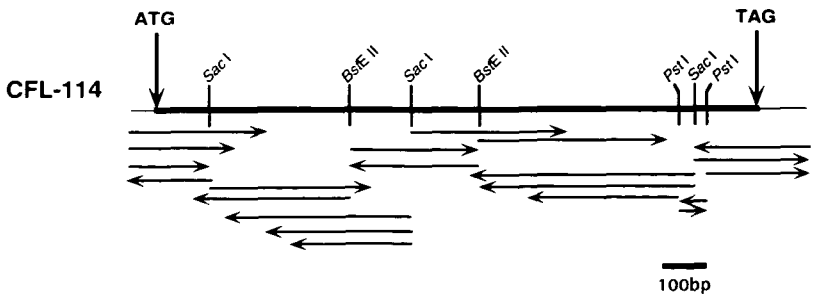

Fig. 1. **Sequencing strategy for dog CYP2D cDNA.** Protein-coding regions are represented by black boxes. The arrows indicate the direction and extent of sequencing. ATG and TAG indicate the initiation and termination codons, respectively.

and cell lysate from cells infected with a dog CYP2D15 recombinant baculovirus (Fig. 2). Sf9 cells infected with wild-type baculovirus and mock-infected cells did not show any cross-reacting proteins.

Spectral analysis revealed that the microsomal fraction of infected cells had a specific P450 content of 0.37-0.56 nmol/mg protein, similar to or higher than the levels of other expressed CYPs from the baculovirus expression system (*33-36*). The microsomal membrane from Sf9 cells infected with wild-type virus did not show a typical difference spectrum of CO-complex of reduced P450 (data not shown).

Catalytic Properties of Dog CYP2D15—We carried out drug-metabolizing studies to characterize the catalytic properties of dog CYP2D15. The microsomal membrane fraction of infected cells (0.4 nmol/mg) was fortified with sufficiently purified rat NADPH P450 reductase (4 units to 30 pmol of P450). Incubation of reaction mixtures containing 4 units of reductase and test drugs with the microsomal membrane from mock-infected Sf9 cells or Sf9 cells infected with wild type did not yielded any detectable quantities of metabolites. Table I shows the drug-metabolizing enzyme activities of the expressed dog CYP2D together with those of male dog liver microsomes.

The results of this study showed that the dog CYP2D15 expressed in Sf9 cell microsomes efficiently catalyzed both bunitrolol 4-hydroxylation and imipramine 2-hydroxylation, which have also been catalyzed by CYP2D subfamilies in rat (*19*) and human (*37*). Cytochrome P450 specific activities of bunitrolol 4-hydroxylase and imipramine 2-hydroxylase catalyzed by the expressed CYP2D15 were about 14-fold and 35-fold higher than those in dog liver microsomes, respectively.

We have found that the expressed dog CYP2D15 in Sf9 cell microsomes also catalyzes imipramine *N*-demethylation at a high rate compared with its activity in dog liver microsomes. Specific *N*-demethylase activity by the expressed CYP2D15 was about 24-fold higher than that in dog liver microsomes.

Debrisoquine 4-hydroxylase activity by the expressed CYP2D15 was 0.05 nmol/min/nmol P450 at the low substrate concentration (50 μM), and its activity in dog liver microsomes at the same substrate concentration was too low to detect. At the high concentration (2 mM), debrisoquine 4-hydroxylase activity by the expressed CYP2D15 was 0.38 nmol/min/nmol P450, which was about 1.5-fold higher than that (0.26 ± 0.09 nmol/min/nmol P450) in dog liver microsomes at the same substrate concentration. Debrisoquine is well known as a substrate for the CYP2D subfamily (*7*), and debrisoquine 4-hydroxylase activity is often used as a marker for CYP2D activity in polymorphism studies. To our knowledge, the present study has been the first to show that the dog liver microsomes are unable to catalyze debrisoquine 4-hydroxylation at the low substrate concentration. We demonstrate that the lack of debrisoquine 4-hydroxylation is not due to the lack of the CYP2D subfamily in dog liver microsomes; instead, dog CYP2D15 has little catalytic ability for debrisoquine 4-hydroxylation at the low concentration of substrate.

Quinine Inhibition Studies—Quinine and quinidine are potent inhibitors of rat CYP2D1/2 (*38*) and human CYP2D6 (*39*), respectively. Our preliminary experiments showed that quinine inhibited more effective inhibitor than quinidine on imipramine 2-hydroxylation and *N*-demeth-

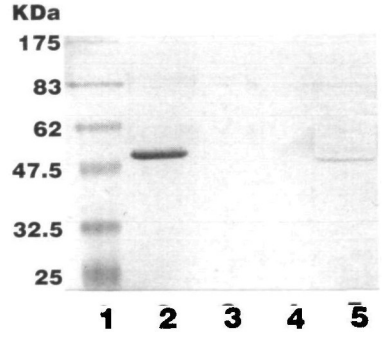

Fig. 2. **Western blot analysis of expressed protein of infected cells.** Microsomal membranes (5 μg) from Sf9 insect cells infected with CYP2D15 recombinant virus (lane 2), those infected with wild-type baculovirus (lane 3), 10 μg of total protein form Sf9 cells (lane 4), and 15.6 μg of dog liver microsomes (lane 5) were separated by SDS-PAGE (10%). CYP2D15 was detected by Western blotting using 0.05 mg/ml of anti-CYP2D15 specific peptide IgG as the first antibody. Molecular weights of the standards are shown by lane 1.

TABLE I. **Catalytic activities of the expressed dog CYP2D15 and liver microsomes from male beagle dogs.**

Reaction[a]	Substrate concentration (μM)	Expressed dog CYP2D[b] (nmol/min/nmol P450)	Dog liver microsomes[c] (nmol/min/nmol P450)
Bunitrolol 4-hydroxylation	10	16.20	1.18 ± 0.10
Imipramine 2-hydroxylation	10	15.11	0.43 ± 0.18
Imipramine *N*-demethylation	10	6.83	0.28 ± 0.03
Debrisoquine 4-hydroxylation	50	0.05	<0.01

[a]Reaction conditions are described in "MATERIALS AND METHODS." [b]Each value is the means of two determinations performed in duplicate. [c]Each value is the mean $\pm$ SD ($n=3$).

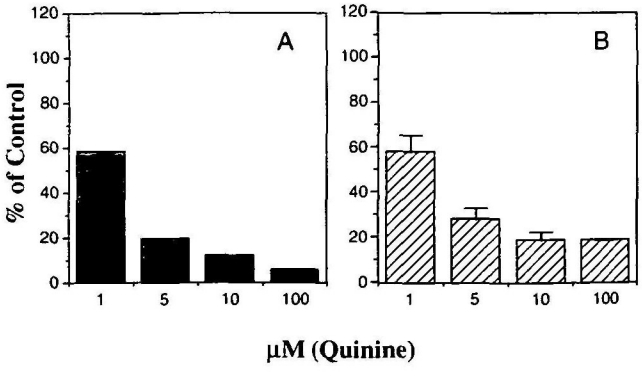

Fig. 3. **Effect of quinine on bunitrolol 4-hydroxylase activity.** The effect of quinine on bunitrolol 4-hydroxylase activities is represented as % of control activity without quinine. (A) Reaction mixtures containing the expressed CYP2D15 were incubated with 10 μM bunitrolol and the indicated amount of quinine. The control activity was 18.3 nmol/min/nmol P450. (B) Reaction mixtures containing dog liver microsomes were incubated with 10 μM bunitrolol and the indicated amount of quinine. The control activity was 1.06 ± 0.05 nmol/min/mg. Error bars indicate the standard deviation ($n=3$).

ylation by dog liver microsomes (data not shown). We therefore chose quinine for our inhibition studies.

In the bunitrolol assay, quinine (100 μM) inhibited 95% of the control 4-hydroxylase activity of recombinant dog CYP2D15, and 80% of the control 4-hydroxylase activity in dog liver microsomes (Fig. 3).

In the imipramine assay, quinine (10 μM) inhibited 2-hydroxylation and *N*-demethylation catalyzed by the recombinant dog CYP2D15 by 71 and 36%, respectively (Fig. 4A). Quinine (10 μM) inhibited control imipramine 2-hydroxylation and *N*-demethylation catalyzed by dog liver microsomes by 58 and 46%, respectively (Fig. 4B). We were unable to obtain reliable data when more than 10 μM quinine was added to the reaction mixture for imipramine metabolism, because the retention time of quinine from the HPLC column was too close to that of 2-hydroxyimipramine.

These results suggest that hydroxylations of bunitrolol and imipramine are mainly catalyzed by dog CYP2D15 in dog liver microsomes. Since quinine inhibited only moderately the activity of imipramine *N*-demethylation by both the expressed CYP2D15 and dog liver microsomes, while it strongly inhibited imipramine 2-hydroxylation, we deduced that either the K_m of imipramine *N*-demethylation by the expressed CYP2D15 was lower than the K_i of quinine inhibition or there are different active sites for imipramine 2-hydroxylation and *N*-demethylation, with quinine acting as a selective inhibitor of the 2-hydroxylation active site. CYPs other than CYP2D15 may also catalyze imipramine *N*-demethylation, because the activity ratio of *N*-demethylation to 2-hydroxylation by the expressed CYP2D15 was 0.45, which was smaller than that (0.65) by dog liver microsomes. In human liver microsomes, CYP3A4 and 1A2 mainly catalyze imipramine *N*-demethylation, while the CYP2D6 in heterologous expression systems is also able to catalyze *N*-demethylation (*40*).

Our quinine inhibition studies suggest that quinine and quinidine are not as potent inhibitors for dog CYP2D15 as for CYP2D isozymes in human and rats, because the effective concentrations of quinine for dog were 10 to 100-fold higher than the concentrations of quinidine for rats and quinine for human. Although quinine may be useful in the study of dog CYP2D15, we need more effective inhibitors for studying the properties of CYP2D15 in dog liver microsomes.

Immunochemical Quantitation of CYP2D15 Specific Contents in Dog Liver Microsomes—Using antibody against a CYP2D15-specific peptide (*26*), we carried out immunochemical quantitation of CYP2D15 specific contents in dog liver microsomes (Fig. 5 and Table II). Microsomes containing expressed CYP2D15 from Sf9 cells were used as the standard CYP2D15 protein. The specific contents of CYP2D15 in dog liver microsomes were 3.18± 0.55% (n=4) of total P450. These data were calculated on the assumption that all the expressed CYP2D15 apoprotein incorporated heme. Since incorporation of heme in expressed CYP is not always 100%, we might have underestimated

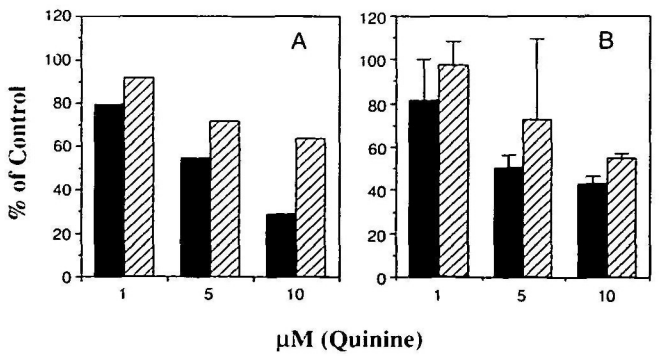

Fig. 4. **Effect of quinine on imipramine 2-hydroxylase and *N*-demethylase activities.** The effect of quinine on imipramine metabolism is represented as % of control activity without quinine. (A) Reaction mixtures containing the expressed CYP2D15 were incubated with 10 μM imipramine and the indicated amount of quinine. The control activities were 20.9 (2-hydroxylase) and 8.57 (*N*-demethylase) nmol/min/nmol P450. (B) Reaction mixtures containing dog liver microsomes were incubated with 10 μM of imipramine and the indicated amount of quinine. The control activity was 0.37±0.06 (2-hydroxylase) and 0.24±0.04 (*N*-demethylase) nmol/min/mg. Error bars indicate the standard deviation (n=3).

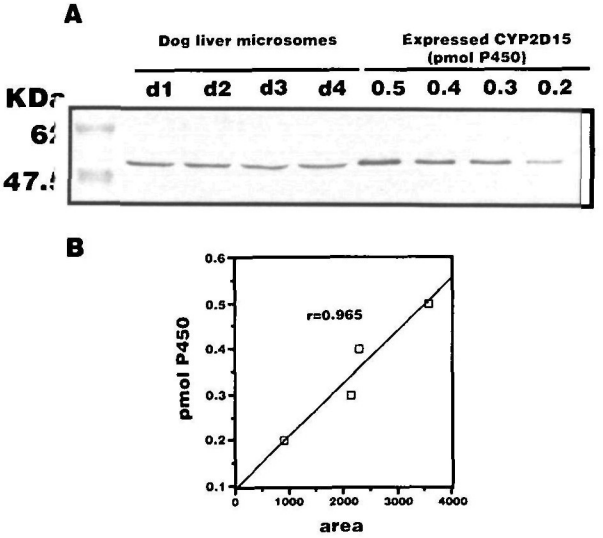

Fig. 5. **Immunochemical quantitation of CYP2D15 in liver microsomes.** (A) Microsomes (13.3 μg) from dog liver (d1-d4) and the indicated amount of the expressed CYP2D15 (0.4 nmol/mg) were subjected to Western blot analysis. Anti-CYP2D15 peptide IgG (0.2 mg/ml) was used as the first antibody. d1, d2, d3, and d4 are the sample numbers of microsomes from four dogs. (B) The area of immuno-reactive bands of the expressed CYP2D15 (in A) were calculated by the NIH image program (*27*). The calibration curve was created by linear regression using a commercial computer application.

TABLE II. **Dog CYP2D15 specific content of total liver P450.**

Dogs	Area[a]	CYP2D15 specific content[b]	
		pmol/mg microsomes protein[c]	% of total P450
d1[d]	2,572	29.22	4.00
d2	2,459	28.24	2.96
d3	2,023	24.48	2.81
d4	2,287	26.76	2.95

[a]Area value was calculated from Western blotting (Fig. 5) by the NIH image program. [b]Specific content was based on the assumption that all the expressed CYP2D15 apoprotein incorporates heme. [c]Total P450 content per mg microsomes from d1, d2, d3, and d4 were 0.73, 0.95, 0.87, and 0.91 nmol/mg, respectively. [d]d1, d2, d3, and d4 indicate sample numbers of microsomes from four dogs.

the CYP2D15 content. Buters *et al.* (*36*) noted that heme incorporation depends on the CYPs isoform. They indicated that ~40% of expressed P450 apoproteins incorporated heme (*36*), except for CYP3A4 apoprotein (~100%) (*35*). If 40% of expressed apoprotein used has incorporated heme, the specific contents of CYP2D15 in dog liver microsomes will correspond to 7.9±1.4% of the total P450, and this is similar to the contents of CYP2D1/2 in rat liver microsomes and CYP2D6 in human liver microsomes (*9, 41*). If we assume that only 20% of the expressed apoprotein used has incorporated heme, the corrected immunochemical quantitation would agree with the results of Sakamoto *et al.*, who indicated that the content of CYP2D15 in dog liver microsomes is about 20% of total P450 contents (*15*).

In summary, we expressed dog CYP2D15 at high levels using a baculovirus expression system. The expressed CYP2D15 had high catalytic activities and a unique substrate specificity from other CYP2D subfamilies. We must emphasize here that debrisoquine 4-hydroxylase activity is not a universal marker for the CYP2D subfamily, because we have found that debrisoquine is a poor substrate of CYP2D15. The expressed CYP2D15 was proven to be useful in studies on the role of CYP2D15 in dog liver drug metabolism, as well as on the structure-function relationships study of the CYP2D subfamily.

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