0026-895X/94/061090-10$03.00/0

MOLECULAR PHARMACOLOGY, 46:1090–1099, 1994.

Catalytic Selectivity and Mechanism-Based Inactivation of Stably Expressed and Hepatic Cytochromes P450 2B4 and 2B5: Implications of the Cytochrome P450 2B5 Polymorphism

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Received June 15, 1994; Accepted September 28, 1994

SUMMARY

Cytochrome P450 (P450) 2B5 was recently found to be functionally distinct from three other rabbit P450 2B forms, based on androstenedione hydroxylase activities. In this investigation, we examined the frequency of the P450 2B5-null phenotype and the functional consequences of polymorphic P450 2B5 expression in hepatic microsomes from phenobarbital-treated rabbits. Four of the 10 animals examined did not have detectable levels of P450 2B5 mRNA and exhibited much lower microsomal androstenedione 15α- and 16α-hydroxylase activities. The 15α-hydroxylase activity was found to correlate ($r = 0.91$) with liver P450 2B5 mRNA. P450 2B4 and 2B5 were stably expressed in human kidney 293 cells to further characterize substrate specificities and to investigate mechanism-based inactivation by phencyclidine. P450 2B4 was 4–16-fold more active than 2B5 towards benzphetamine, 7-ethoxycoumarin, methylenedioxybenzene, and pentoxyresorufin. Benzyloxyresorufin *O*-debenzylase activity was 160-fold higher for P450 2B4 than P450 2B5. Anti-P450 2B4 IgG inhibited benzyloxyresorufin *O*-debenzylation nearly completely in untreated and phenobarbital-induced liver microsomes. Phencyclidine selectively inactivated P450 2B4, compared with 2B5, in both human kidney 293 cell and liver microsomes. Poor inactivation of P450 2B5 by phencyclidine was found to be a result of its low maximal rate constant. Results of this study establish the idea that the metabolic consequences of phenobarbital induction depend on the potential of animals to express functionally variant P450 2B forms. Furthermore, we conclude that one or more of the 11 amino acid differences between these highly related P450 forms are critical to their substrate specificities and selective inactivation.

P450s comprise a superfamily of membrane-bound enzymes (1) with a broad capacity for oxidative metabolism of endogenous and foreign chemicals. Multiple P450 subfamilies are present within a single mammalian species. The levels of P450 enzymes in a tissue such as liver can be controlled by a variety of regulatory mechanisms, including hormonal up- or down-regulation (2) and induction by exposure to foreign compounds (3, 4). Frequently, dramatic quantitative and qualitative changes in the P450 profile can occur after treatment with inducing chemicals such as PB. The changes in the levels of individual P450 enzymes can alter the metabolic disposition of a drug or environmental chemical and can therefore be an important determinant of pharmacological efficacy or toxicity.

PB is a prototype for a variety of structurally diverse chemicals that are able to induce P450 2B enzymes in several mammalian species. Advances in cDNA cloning and protein expression technology have begun to reveal new insights into the expression patterns and catalytic activities of individual P450 2B forms. The study of P450 2B forms in rats, rabbits, and mice is complicated by both the presence of multiple, closely related forms and their differential expression (5–7). Similarly to rat P450 2B1 and P450 2B2, rabbit P450 2B4 and P450 2B5 exhibit remarkably different tissue expression patterns. Rat P450 2B2 and rabbit P450 2B5 are constitutively expressed at low levels in liver but are not found in lung tissue. P450 2B1 and P450 2B4 are both expressed constitutively in lung. The levels of P450 2B enzymes in rats and rabbits are dramatically increased in liver after treatment with PB but are not changed in lung. Additional complexity is introduced by the presence of multiple allelic variants that have been shown to characterize the *CYP2B1* and *CYP2B2* loci (5, 8, 9). P450

This research was supported in part by Grant ES03619 (to J.R.H.) from the National Institutes of Health.

ABBREVIATIONS: P450 or CYP, cytochrome P450; PB, phenobarbital; DMEM, Dulbecco's modified Eagle's medium; SDS, sodium dodecyl sulfate; NBT, nitroblue tetrazolium; BCIP, 5-bromo-4-chloro-3-indolyl phosphate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MDB, methylenedioxybenzene; MTBE, methyl-*t*-butyl ether; PBS, phosphate-buffered saline; PCP, phencyclidine; BROD, benzyloxyresorufin *O*-debenzylase; ECOD, ethoxycoumarin *O*-deethylase; kb, kilobase(s); CMV, cytomegalovirus; HK, human kidney; PAGE, polyacrylamide gel electrophoresis.

2B1 and 2B2 generally exhibit similar substrate specificities but differ in turnover number. The metabolism of dimethylbenzanthracene is an exception to this general trend, where microsomal P450 2B2 favors 12-hydroxylation, as opposed to 7-hydroxylation catalyzed by P450 2B1 (10). Regio- or stereochemical differences in steroid hydroxylation have been demonstrated for some of the rat P450 2B variants, suggesting that allelic variation may have some physiological significance (11, 12).

Rabbits have been shown to express at least four P450 2B forms, exhibiting >97% amino acid sequence identity (6, 13). Other cDNA sequences have been published for rabbit P450 2B forms, but they appear to be allelic variants rather than additional genes (1, 14). The cDNAs studied in our laboratories were designated 2B-B0, 2B-B1, 2B-B2, and 2B-Bx, based on restriction analysis and sequencing. The 2B-B0 cDNA was shown to have a deduced amino acid sequence identical to that reported for the purified P450 "LM2" enzyme (15), and we have therefore designated the gene product as P450 2B4. The 2B-B2 cDNA is designated here as P450 2B5, based on its tissue-specific expression.[1] The possibility of at least five different P450 2B phenotypes in rabbit liver was recently described (13), based on mRNA analysis in various tissues. Results from those investigations showed that some animals may lack the ability to express the 2B5 form.

Functional characterization of variant P450 2B forms is important for several reasons, including the potential 1) to pinpoint the structural elements that might alter catalytic function and 2) to investigate the metabolic and toxicological consequences of individual variation and organ specificity in P450 expression patterns. The presence of multiple, closely related, P450 2B forms in liver tissue has made protein purification techniques less desirable as a means to characterize the structure and function of individual rabbit P450 2B forms. In fact, most preparations of P450 LM2 purified from rabbit liver likely contain multiple P450 2B forms. The four rabbit P450 2B forms were recently studied in our laboratories by using transient expression in COS-7 cells. Three of these forms, P450 2B4, 2B-B1, and 2B-Bx, were functionally indistinguishable, based on their steroid hydroxylase activities (13). In contrast, the P450 2B5 form was found to catalyze the hydroxylation of androstenedione with distinct regio- and stereospecificity (13, 16). Androstenedione 15α-hydroxylase activity was proposed as a potential specific marker of P450 2B5 activity. Because the P450 2B4, 2B-Bx, and 2B-B1 forms appear to be functionally identical, phenotypes with one or more of these three P450 2B variants would not be expected to be functionally different. In contrast, rabbits that express the 2B5 form may demonstrate altered hepatic metabolism, compared with rabbits that do not express this form.

Benzphetamine, 7-ethoxycoumarin, and certain 7-alkoxyresorufins are frequently used to determine the activity of P450 2B enzymes in microsomes and purified preparations (17–19). Androstenedione and testosterone have been used more recently as specific probes, because they are hydroxylated with pronounced regio- and stereoselectivity by many P450 enzymes (20). P450 2B enzymes are also involved in the metabolism of other compounds, such as MDB and PCP. Metabolism of MDB by P450 2B produces catechol, a potentially toxic metabolite (21). MDB metabolism can also inhibit drug metabolism due to the formation of a metabolite intermediate complex (22). PCP, a frequent drug of abuse with significant toxicological risk, can also inhibit drug metabolism due to the metabolism-dependent inactivation of P450 2B enzymes (23). Based on our previous investigations showing the regio- and stereoselective hydroxylation of androstenedione by P450 2B5, compared with the other rabbit 2B forms, we sought to evaluate the selectivity of these enzymes toward commonly studied P450 2B substrates.

In this investigation, we examined the incidence of the P450 2B5-null phenotype and the functional consequences of the P450 2B5 polymorphism in rabbit hepatic microsomes. We also report here on a novel stable expression system for P450 2B4 and 2B5, using HK 293 cells. This expression system has allowed us to further evaluate the catalytic selectivity of these highly related enzymes and to identify a specific substate for P450 2B4. Stably expressed P450 2B4 and 2B5 and specific markers for their activities in microsomes allowed us to investigate the selectivity of PCP as a mechanism-based inactivator of these enzymes.

Experimental Procedures

Materials. DNA isolation kits were purchased from Qiagen (Chatsworth, CA). Restriction endonucleases, DNA modification enzymes, DMEM, penicillin/streptomycin, and Geneticin 228 (G418 sulfate) were purchased from GIBCO-BRL (Bethesda, MD). NuSerum IV was purchased from Collaborative Biomedical (Bedford, MA). Growth media for *Escherichia coli* were obtained from Difco (Detroit, MI). The plasmid pRc/CMV was purchased from Invitrogen (San Diego, CA). A control cDNA probe for glyceraldehyde-3-phosphate dehydrogenase was obtained from Clontech (Palo Alto, CA). γ-End labeling and nick translation kits were purchased from Boehringer Mannheim (Indianapolis, IN). All SDS-PAGE reagents were purchased from Bio-Rad (Richmond, CA). [4-^{14}C]Androst-4-ene-3,17-dione and [^{32}P]ATP were obtained from New England Nuclear (Boston, MA). Thin layer chromatography plates [silica gel, 250 μm, Si 250 PA (19C)] were purchased from Baker Chemicals (Phillipsburg, NJ). NADPH, androstenedione, 16α-hydroxyandrostenedione, 7-ethoxycoumarin, umbelliferone, benzphetamine, resorufin, pentoxyresorufin, and benzyloxyresorufin were purchased from Sigma Chemical Co. (St. Louis, MO). MDB, catechol, and sesamol were obtained from Aldrich (Milwaukee, WI). All other reagents and supplies were obtained from standard commercial sources.

Animals, treatments, and preparation of microsomes. Male New Zealand White rabbits (1.5–2 kg) were purchased from Hazleton Laboratories (Denver, PA) and were administered PB (0.1%, w/v) in the drinking water for 5 days. The rabbits were killed by pentobarbital overdose. Liver tissue was removed and frozen in liquid nitrogen for subsequent preparation of RNA. Separate pieces of liver were processed immediately for preparation of microsomes, using previously described methods (24). Microsomal suspensions were stored in 10 mM Tris-acetate, pH 7.4, 0.1 mM EDTA, 20% glycerol, at −80°.

Northern analysis of total hepatic RNA. Total RNA was prepared from 0.1–0.3 g of liver tissue using an acid guanidinium isothiocyanate-phenol/chloroform extraction method (25, 26). Lithium chloride was used to remove glycogen from the initial RNA pellet before final purification. Samples of total RNA (20 μg) from each of the 10

[1] The nomenclature for P450 2B forms in rabbits is as follows. The nomenclature described by Nelson *et al.* (1) lists two 2B forms (2B-B0 and 2B-B1) as allelic variants of *CYP2B4* and 2B-B2 as a product of *CYP2B5*. The presence of the Bx form was established after publication of this nomenclature. We have chosen to refer to the 2B-B2 form as P450 2B5 and the 2B-B0 form as 2B4, because of its identical deduced amino acid sequence, compared with that of purified P450 2B4 (15). In this paper, we continue to refer to the other two rabbit 2B forms as 2B-B1 and 2B-Bx, because the gene assignments of the four forms are still uncertain at this time. For the hepatic microsomal activities discussed in this paper, the term "P450 2B4" refers to the combined activity of P450 2B4, 2B-B1, and 2B-Bx, because of their catalytic similarity. Heterologously expressed P450 2B4 refers to the single form encoded by the B0 cDNA.

PB-treated rabbits were electrophoresed on 1% agarose gels containing formaldehyde. Separated RNA was transferred to positively charged nylon membranes (Boehringer Mannheim). The nylon membranes were washed in 2× standard saline citrate (0.3 M NaCl, 0.030 M sodium citrate, pH 7.0) and UV-cross-linked for 35 sec. Membranes were incubated for 3 hr at 48° in hybridization solution (5× SSPE (0.9M NaCl, 50 mM NaH_2PO_4, and 50 mM EDTA), pH 7.4, 5× Denhardt's solution, 0.1% sodium pyrophosphate, 0.1% SDS, 50% formamide, 4 μg/ml of sheared salmon sperm DNA). The blots were probed overnight at 48° or 55° in hybridization solution containing 5′-^{32}P-end-labeled oligonucleotides (2B4 probe, 5′-AATGACCAGGGGTCCCTGTGGCC-3′-OH; 2B5 probe, 5′-ACGACCAGGGCGGTGCGGTGGGG-3′-OH) specific for the P450 2B-BO and -B2 forms, respectively. Hybridized blots were washed three times for 30 min in 1× standard saline citrate/0.2% SDS at the hybridization temperature and were then subjected to autoradiography at −70°.

Transient expression of the rabbit P450 2B forms in COS-7 cells. The cDNAs encoding the four known rabbit P450 2B forms (2B4, 2B-Bx, 2B-B1, and 2B5) were transfected into COS-7 cells using DEAE-dextran, as described previously (11). COS-7 cells were grown in monolayer culture at 37° in 5% CO_2, in DMEM supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin. After transfection, COS cells were maintained in DMEM with 5% fetal bovine serum. Microsomes were prepared approximately 72 hr after transfection and were stored at −70° until used.

Stable expression in HK 293 cells. HK 293 cells were grown at 37° in 5% CO_2 in DMEM containing 10% NuSerum IV. The cDNAs encoding the 2B4 and 2B5 forms were bidirectionally subcloned, using *Hind*III, from the COS cell expression vector pBC12BI into the multiple-cloning site of the eukaryotic expression vector pRc/CMV. The pRc/CMV vector contains the CMV promoter for high level expression and a neomycin resistance gene for selection of cells that have incorporated the plasmid genes. Insertion of the cDNAs into the pRc/CMV vector was determined by polymerase chain reaction. The correct orientations of the 2B4 and 2B5 cDNAs in pRc/CMV were checked using *Apa*I digestions and fragment length analysis. Plasmid DNAs containing cDNA inserts in the correct orientation were isolated and purified for HK 293 transfections using Qiagen columns.

The resulting plasmids, pRcB0 and pRcB2, were transfected into HK 293 cells using a calcium phosphate precipitation method (Transfinity kit; GIBCO-BRL). After a 24-hr incubation with precipitated plasmid DNA, the cells were washed and grown for an additional 24 hr in fresh DMEM containing 10% NuSerum IV. On the third day, the cells were split 1/20 and 1/50 into DMEM containing 10% NuSerum IV and 0.8 mg/ml G418 sulfate for selection of cells containing the neomycin resistance gene. Resistant cell foci were isolated using cloning rings, after an 18–21-day selection period. Isolated cell foci were expanded into six-well plates containing the selection medium. Cell lines expressing high levels of P450 2B4 or 2B5 were chosen for subculture using immunoblotting and ECOD or androstenedione 15α-hydroxylase activity assays, respectively. The cells were routinely subcultured once each week. Cells were detached using fresh serum-free culture medium. Trypsin was used, after gentle rinsing of the plates with PBS, to detach cells and break up cell aggregates on every third passage.

HK 293 cells could be removed from culture dishes without digestive enzyme treatment by washing of the plates with cold PBS, pH 7.4. Cells were pelleted by refrigerated centrifugation in PBS. The pelleted cells from eight to 16 plates (15-cm diameter) were sonicated on ice for 15 sec (twice) in 0.25 M sucrose, 1 mM EDTA. Cell debris was removed by centrifugation at 10,000 × *g* for 5 min. Microsomes were pelleted by centrifugation at 101,000 × *g* for 30 min, resuspended in buffer containing 10 mM Tris acetate, pH 7.5, 0.1 mM EDTA, and 20% glycerol, and stored at −70°.

Catalytic activities of P450 2B4 and 2B5. Androstenedione hydroxylase activities were determined by incubating 25 μM [^{14}C]-androstenedione for 5 min with 25 μg of liver microsomes or for 30 min with 200 μg of HK 293 microsomes. Incubations included 1 mM NADPH and incubation buffer (50 mM HEPES, pH 7.5, 0.1 mM EDTA, with 15 mM $MgCl_2$ for liver microsomes or 3 mM $MgCl_2$ for HK 293 microsomes). Exogenous reductase was not added to HK 293 cell microsomal incubations.

Benzphetamine *N*-demethylation was determined by assaying for formaldehyde formation (27). Benzphetamine incubations were carried out for 30 min using 1 mM substrate, 1 mg of HK 293 microsomal protein, 33 mM phosphate buffer, pH 7.4, 5 mM $MgCl_2$, and an NADPH-generating system of 10 mM glucose-6-phosphate, 1 mM $NADP^+$, and 1 unit/ml glucose-6-phosphate dehydrogenase.

ECOD activity was determined by incubating 200 μg of HK 293 microsomal protein for 5–15 min with 0.3 mM 7-ethoxycoumarin, incubation buffer (as described for androstenedione incubations), and 1 mM NADPH. The reactions were terminated by addition of 0.1 ml of 2 N HCl, and the reaction mixtures were extracted with 2 ml of MTBE. The MTBE layer was back-extracted against 30 mM sodium borate. Hydroxycoumarin was determined fluorimetrically at 454 nm, with an excitation wavelength of 366 nm (28).

The formation of catechol from MDB was measured by incubating microsomal preparations (1 mg of protein) with 1 mM MDB and 50 units of superoxide dismutase, in incubation buffer (as described for androstenedione incubations), for 15 min at 37°. The reaction products catechol and sesamol were separated and quantitated by reverse phase high performance liquid chromatography, with UV detection at 231 nm. Incubations (0.5 ml) were extracted with 2 ml of MTBE. Redissolved sample residues were injected onto a 250- × 4.6-mm Zorbax SB-C8 column (MacMod, Chadds Ford, PA) with a mobile phase of 20% acetonitrile/water, at a 1.5 ml/min flow rate. Retention times for catechol, sesamol, and MDB were 4.5, 6.5, and 10 min, respectively.

Pentoxyresorufin (10 μM) or benzyloxyresorufin (10 μM, 250-fold dilution from a dimethylsulfoxide stock solution) was incubated for 15 min in buffer (described above for androstenedione incubations) with 0.1–0.2 mg of HK 293 microsomes. Incubations were started by the addition of 1 mM NADPH. Liver microsomes (20 μg) were incubated, under the same conditions, for 2 min after the addition of NADPH. Reactions were stopped by the addition of 1.5 ml of methanol. Precipitated protein was removed by centrifugation. The fluorescent product resorufin was measured with an excitation wavelength of 550 and an emission wavelength of 585 nm.

Immunoblot analysis. SDS-PAGE (10%) was conducted using standard conditions (29). Separated proteins were electrophoretically transferred to nitrocellulose membranes as described previously (16). After blocking, the nitrocellulose membranes were incubated with goat anti-rabbit lung 2B4 IgG (20 μg/ml, in 3% nonfat dry milk) and then with alkaline phosphatase-conjugated rabbit anti-goat IgG. IgG-bound protein was detected using the alkaline phosphatase-catalyzed NBT/BCIP reaction (SigmaFast; Sigma Chemical Co.).

Immunoinhibition experiments. Untreated or PB-induced microsomes were incubated at room temperature with preimmune IgG or goat anti-rabbit lung 2B4 IgG (10 μg/μg of microsomal protein) for 30 min before addition of the incubation components for assay of androstenedione hydroxylase or BROD activity as described above.

Inactivation of P450 2B4 and 2B5 by PCP. For inactivation experiments with heterologously expressed P450 2B4 and 2B5, primary incubations contained 0.1 mM PCP with 5 mg/ml HK 293 microsomes, 50 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 0.1 mM EDTA, and 1 mM NADPH, at 37°. The mixtures were preincubated with the inhibitor for 3 min before the addition of NADPH to start the reaction. Aliquots containing 200 μg of expressed protein were removed at various time points and added to secondary incubations containing 50 mM HEPES, pH 7.5, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 mM NADPH, and 0.3 mM 7-ethoxycoumarin (P450 2B4) or 25 μM androstenedione (P450 2B5). In PB-induced hepatic microsomes, various concentrations of inhibitor were preincubated to 37° with liver microsomes (1 mg/ml protein) in buffer containing 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, and 0.1 mM EDTA. Reactions were started by addition of NADPH to a final concentration of 1 mM. Aliquots containing 20 or 25 μg of microsomal

protein were transferred at various times to secondary incubations containing 25 μM [^{14}C]androstenedione or 10 μM benzyloxyresorufin in 50 mM HEPES, pH 7.5, 15 mM $MgCl_2$, 0.1 mM EDTA. Secondary incubations were allowed to proceed for 5 min (for the expressed 2B forms) or 2 min (for hepatic microsomes) before the reaction was quenched. Metabolite formation for each substrate was assayed as described above.

Data analysis. The kinetic parameters for inactivation of microsomal P450 2B4 and 2B5 were estimated using a nonlinear regression analysis program (ENZFITTER; Biosoft, Cambridge, UK).

Miscellaneous methods. P450 content was determined by measuring the reduced carbon monoxide difference spectrum (absorption coefficient of 91 mM^{-1} cm^{-1}) between 450 and 490 nm (30). NADPH-P450 reductase was assayed in each cell line by measuring reduction of cytochrome *c*, using a standard colorimetric method (31). Microsomal protein was measured using bicinchoninic acid reagent (32), with bovine serum albumin standards.

Results

Effects of polymorphic P450 2B5 expression on androstenedione metabolism. Previous investigations in our laboratories suggested that rabbits treated with PB can exhibit at least five P450 2B phenotypes in liver and that some animals may not express P450 2B5 (13). Androstenedione 15α- and 16α-hydroxylations were identified in yeast and COS cell expression systems as activities of P450 2B5, whereas the other three rabbit P450 2B forms catalyze 16β-hydroxylation, with little 16α- and no 15α-hydroxylase activity. In this study, we sought to 1) determine the incidence of the P450 2B5-null phenotype, 2) determine the effect of individual variation in P450 2B5 expression on the metabolism of androstenedione in hepatic microsomes, and 3) substantiate the use of androstenedione 15α-hydroxylase activity as a marker of P450 2B5 activity. Total hepatic RNA and microsomal fractions were prepared from 10 PB-treated rabbits. The total P450 levels in the induced microsomal fractions varied from 2.3 to 3.1 nmol of P450/mg of protein. On a Western blot, little variation was observed in the levels of immunoreactive protein among the induced animals, using a polyclonal anti-P450 2B4 IgG that detects all P450 2B forms in rabbits (Fig. 1). The four rabbit P450 2B forms are not separable by conventional SDS-PAGE methods, so the contribution of each form to the total P450 2B response could not be assessed on the immunoblot.

Northern blot analysis was performed to evaluate the incidence of the null phenotype, i.e., animals devoid of P450 2B5. Blots hybridized with oligonucleotides that specifically detect 2B4 or 2B5 mRNA each contained a single major band, of approximately 2.4 kb (Fig. 2A).[2] Additional minor bands at

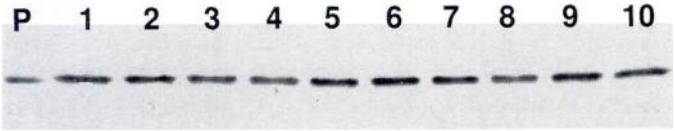

Fig. 1. Immunoblot of microsomes prepared from 10 individual PB-treated rabbit livers (*lanes 1-10*); *lane P*, 0.3 pmol of purified P450 2B4. Microsomal protein (0.3 μg) from each sample was separated on a denaturing 7.5% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane. P450 2B forms were detected with goat anti-rabbit P450 2B4, rabbit anti-goat IgG conjugated to alkaline phosphatase, and BCIP/NBT color reagents.

~2.7 and 3.2 kb were also detected using the 2B5 oligonucleotide. The P450 2B5 oligonucleotide hybridized with mRNA in only six of the 10 rabbit livers, whereas four animals contained no detectable 2B5 mRNA, indicating that the null phenotype is common in rabbits. RNA isolated from one animal (rabbit 4) that expressed P450 2B5 did not hybridize with the P450 2B4-specific oligonucleotide, whereas three animals had considerably weaker 2B4 bands than did the remaining animals. Because P450 2B5 has never been shown to be expressed in liver without the presence of other P450 2B forms, rabbit 4 likely also expressed the P450 2B-B1 and/or 2B-Bx forms. The rabbit 2B-B1 and 2B-BX forms have catalytic activities similar to that of P450 2B4 (13). Therefore, any functional changes that might occur due to differences in the expression levels of P450 2B4 in microsomes would depend on whether the animal also expressed the 2B-B1 or 2B-Bx form.[4]

The regio- and stereoselective hydroxylation of androstenedione was monitored in the rabbit hepatic microsomes, to determine the functional consequences of polymorphic expression of P450 2B5. Significant interanimal variation was clearly evident in the androstenedione metabolite profiles for the 10 PB-treated animals (Fig. 2B). Whereas little variation was observed between animals for androstenedione 6β- and 16β-hydroxylase activities, the 15α- and 16α-hydroxylase activities were highly variable. Four animals (rabbits 2, 5, 6, and 10) exhibited very low 15α- and 16α-hydroxylase activity.[3] The remaining six animals had much higher 15α- and 16α-hydroxylase activities. The androstenedione 15α-hydroxylase activities in each sample were significantly correlated ($r = 0.91$) with the amount of 2B5 mRNA estimated by laser densitometry (Fig. 3). A correlation could also be made between 2B5 expression and the 16α-hydroxylase activity in microsomes, although P450 forms other than those in the 2B subfamily also contribute to this steroid hydroxylase activity (16, 18). Androstenedione 15α-hydroxylase activity might be used in the future as a microsomal marker of P450 2B5 expression in liver when investigators are interested in the contribution of this P450 2B form to the metabolism of drugs or other chemicals.

Stable heterologous expression of P450 2B4 and 2B5. Stable expression of P450 2B4 and 2B5 was used as a means to study the catalytic activities of these structurally similar but functionally different 2B forms in more detail, by providing a continuous source of the individual enzymes. HK 293 cells were chosen for stable expression of P450 2B4 and 2B5 because of the available vectors for high level expression and the presence of sufficient NADPH-P450 reductase to support P450 catalysis.

Androstenedione hydroxylase activities were not detected in the native HK 293 cell line in initial evaluations of these cells as a candidate for P450 expression (data not shown). The cDNAs encoding P450 2B4 and 2B5 were transfected into HK 293 cells using the pRc/CMV expression vector. After a period of stringent selection with a neomycin analog, resistant cell foci were individually expanded. *In situ* assays for steroid

[2] Complementary 23-mer oligonucleotide probes (*CYP2B4* bases 1495–1517 and *CYP2B5* bases 1498–1520) specific for P450 2B4 and P450 2B5 were synthesized based on sequence regions 3′ to their stop codons. Much of the 2B5 probe sequence corresponds to deletions in the 3′ flanking regions of the P450 2B4, 2B-B1, and 2B-Bx forms (13). The 2B4 oligonucleotide does not detect the 2B-B1 and 2B-Bx forms.

[3] The low level (0.04 nmol/min/mg) of 15α-hydroxylase activity in the livers that were devoid of 2B5 mRNA was only partially inhibited by the addition of anti-P450 2B4 IgG (data not shown). Additionally, lung microsomes (P450 2B5 is not expressed in lung) were found to have only trace androstenedione 15α-hydroxylase activity (8 pmol/min/mg). These results suggest that the low residual androstenedione 15α-hydroxylase activity is due to P450s other than those of the 2B subfamily. Because of this low residual activity, it will be important to use liver microsomes from rabbits with high 15α-hydroxylase activity (0.4–0.5 nmol/min/mg) in future studies of P450 2B5.

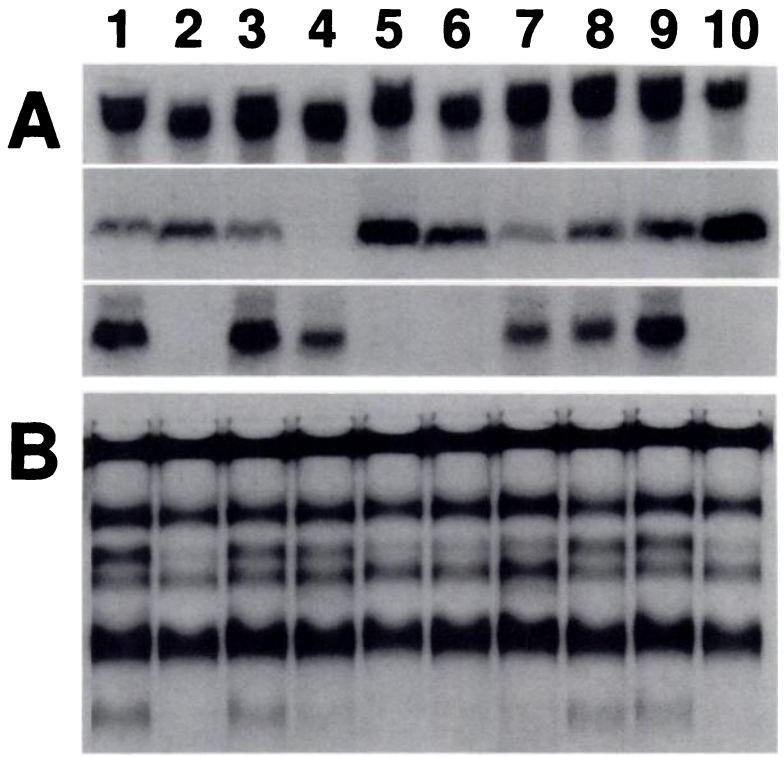

Fig. 2. A, Northern blots of total RNA recovered from 10 individual PB-treated rabbit livers (*lanes 1-10*). Total liver RNA (20 μg) was separated on agarose gels and hybridized with ^{32}P-labeled oligonucleotides specific for the 2B4 and 2B5 forms of P450 2B. The blot that was originally hybridized with the 2B5 oligonucleotide probe was rehybridized (*Co*) with a cDNA probe encoding glyceraldehyde-3-phosphate dehydrogenase (1.2 kb), to control for differences in total RNA loading. B, Autoradiogram of hydroxylated androstenedione metabolites from incubations with microsomes from 10 individual rabbit livers. Microsomes (25 μg of protein) were incubated with 25 μM ^{14}C-labeled substrate for 5 min before the incubation was stopped by the addition of tetrahydrofuran. Aliquots (one third of the total volume of the sample) were separated on a thin layer chromatography plate.

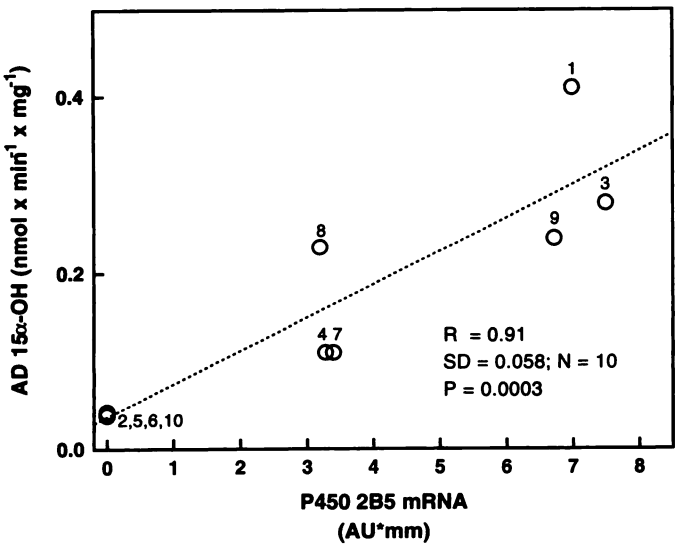

Fig. 3. Correlation between androstenedione (*AD*) 15α-hydroxylase activity and P450 2B5 mRNA in PB-treated rabbit livers. The level of mRNA hybridized with a 2B5-specific oligonucleotide was estimated using laser densitometry. Liver RNA from four animals (rabbits 2, 5, 6, and 10) did not hybridize with the P450 2B5-specific oligonucleotide. These four animals had microsomal 15α-hydroxylase activity of 0.04 nmol/min/mg. *AU*, absorbance units.

hydroxylase (2B5-containing cells) or ECOD (2B4-containing cells) were performed on 40–50 clones containing each recombinant P450 2B form, to facilitate the subculture of those cell lines exhibiting the highest enzyme activity. A 5–10-fold variation in enzyme activity was observed between different clonal cell lines when substrate was incubated *in situ*. This difference in activity was probably due to the copy number of each gene incorporated into the cell genome, although this possibility was not investigated. The three or four cell lines that exhibited the highest activity were subcultured. Microsomes were prepared from the subcultured cell lines for further analysis. For each cell line, the amount of protein detected on immunoblots was found to correlate well with catalytic activity measured in microsomal fractions.

HK 293 microsomal proteins were resolved on SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and detected with a polyclonal antibody to rabbit P450 2B4 and an alkaline phosphatase-catalyzed color reaction (Fig. 4). A single protein band was detected for each P450 2B form, whereas no immunoreactive protein was detected in sham-transfected cells (Fig. 4, *lane 6*). Densitometric estimation of the amounts of each P450 2B form in microsomes was performed using multiple standard levels of purified P450 2B4. P450 2B5 was expressed at approximately 100 pmol/mg of microsomal protein, a relatively high level in mammalian expression systems. The 2B4 form was expressed at approximately 30 pmol/mg in this cell line. Degradation to lower molecular weight fragments did not appear to be a factor contributing to the difference in maximal expression levels for each form. Nearly constant expression levels for the two P450 2B proteins were observed over a period of approximately 9 months, with weekly passage of cells. The levels and stability of expression, may make the HK 293 cell line an excellent candidate for stable expression of other P450 enzymes.

Catalytic activities of stably expressed P450 2B4 and 2B5. Until recently, little was known about the multiplicity of P450 2B forms and the functional uniqueness of P450 2B5. Our objective in further characterizing the substrate specificities of P450 2B4 and 2B5 was to measure their contributions to the metabolism of some of the substrates most commonly used to measure the activity of purified preparations of P450 2B4 and to monitor the induction or inhibition of P450 2B enzymes in microsomes. Additionally, we sought to identify a

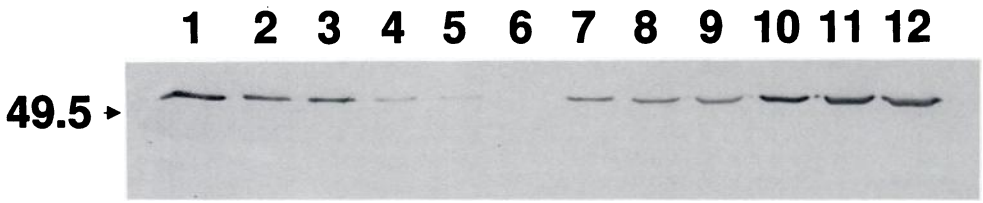

Fig. 4. Immunoblot analysis of P450 2B4 and 2B5 expressed in HK 293 cell microsomes. Microsomal protein from each sample was separated by 10% SDS-PAGE and transferred to a nitrocellulose membrane. The expressed proteins were detected with goat anti-rabbit P450 2B4, rabbit anti-goat IgG conjugated to alkaline phosphatase, and BCIP/NBT color reagents. *Lanes 1-5*, 1.0, 0.3, 0.3, 0.1, and 0.05 pmol of purified P450 2B4; *lane 6*, 20 μg of sham-transfected HK 293 cell microsomes; *lanes 7-9*, 5, 10, and 20 μg of microsomal protein from HK 293 cells expressing P450 2B4; *lanes 10-12*, 5, 10, and 20 μg of microsomal protein from HK 293 cells expressing P405 2B5. Densitometric analysis of the expressed proteins and the 2B4 standards showed the amounts of 2B4 and 2B5 in HK 293 cell microsomes to be approximately 30 and 100 pmol/mg of protein, respectively.

substrate that could be used to monitor P450 2B4 activity (like the androstenedione 15α-hydroxylase activity for P450 2B5) without interference from P450 2B5 or P450s from other subfamilies.

To validate the HK 293 expression system, the metabolism of androstenedione by P450 2B4 and 2B5 expressed in HK 293 cells was compared with that observed in the COS expression system (13). The regio- and stereospecific hydroxylations of androstenedione observed after expression in HK 293 cells were similar to those observed for the 2B forms expressed in COS cells. P450 2B4 expressed in HK 293 cells catalyzed the hydroxylation of androstenedione exclusively at the 16-position, with a β/α ratio of about 10 (Table 1). This $16\beta/\alpha$ hydroxylation ratio is slightly different from that reported for COS-expressed P450 2B4 ($\beta/\alpha = 5$). This difference is probably due to the inaccuracy involved in measuring the very low 16α-hydroxylase activity for 2B4 in these expression systems. P450 2B4 purified either from lung, where P450 2B5 is not present, or from liver tissue that does not contain 15α-hydroxylase activity has $16\beta/\alpha$ ratios of 12–14 (13). HK 293-expressed P450 2B5 catalyzed 16α-, 15α-, and 6β-hydroxylation, whereas 16β-hydroxylation was not observed for this form. 6β-Hydroxylase activity was occasionally observed in COS cells expressing P450 2B5 but was not previously quantitated due to high background levels in the native cell line. As previously shown with the P450 2B forms expressed in COS cells, the stably expressed P450 2B4 had much higher ECOD activity than did 2B5. The 14-fold difference in activity for the 2B4 form could make 7-ethoxycoumarin a good candidate as a selective 2B4 marker. However, the use of 7-ethoxycoumarin as a specific 2B4 marker in liver microsomes is precluded by the fact that P450s from other families and subfamilies also metabolize this compound (18).

Benzphetamine *N*-demethylation, probably the most common reaction used to determine P450 2B activity in microsomes and reconstituted systems, was catalyzed by both P450 2B4 and 2B5 (Table 2). The *N*-demethylase activity of 2B4 was approximately 4-fold higher than that of 2B5. Therefore, benzphetamine will probably continue to be a useful marker of P450 2B4 function in lung microsomes, where 2B5 is not expressed. In liver tissue, however, the significant contribution of 2B5 to benzphetamine *N*-demethylation makes this marker less useful without determination of the P450 2B phenotype of the donor animal. Similarly to the selectivity of the two P450 2B forms towards benzphetamine, 2B4 catalyzed the dealkylation of pentoxyresorufin at a 5-fold higher rate than did the 2B5 form. This substrate has frequently been used in rat liver microsomes to determine the induction of P450 2B. Based on these findings, some consideration should be given to the multiplicity of P450 2B forms in rabbits when using pentoxyresorufin to monitor PB-type induction. P450 2B4 expressed in HK 293 cells catalyzed the formation of catechol from MDB, whereas the expressed P450 2B5 metabolized MDB at a 16-fold lower rate. Therefore, one might expect MDB to be metabolized in all cell types that express P450 2B4 and be little influenced by the polymorphic expression of P450 2B5 in liver.

The HK 293-expressed P450 2B4 was extremely selective, compared with the 2B5 form, towards benzyloxyresorufin (160-fold difference). In an effort to determine whether the rabbit P450 2B-BX and 2B-B1 forms also catalyze the *O*-dealkylation of benzyloxyresorufin, microsomes from COS-7 cells expressing each of the four P450 2B forms were incubated under reaction conditions identical to those used for the HK 293-expressed enzymes. The P450 2B4, 2B-Bx, and 2B-B1 forms expressed

TABLE 1

Androstenedione hydroxylase activities of P450 2B4 and P450 2B5 expressed in HK293 cells

Microsomes (0.2 mg) were incubated with [^{14}C]androstenedione (25 μM substrate) in the presence of 1 mM NADPH. Reactions were carried out for 30 min at 37°. Hydroxylated metabolites and unmetabolized androstenedione were separated by thin layer chromatography. The activities are the average of duplicate determinations, using pools from the HK 293 cell lines. The numbers in parentheses are turnover numbers (pmol/min/nmol) based on densitometric estimation of the amount of protein on a Western blot, using goat anti-P450 2B4.

Expressed P450 2B form	Androstenedione hydroxylase activity			
	15α-OH	16α-OH	16β-OH	6β-OH
	pmol/30 min/mg of protein			
Sham	ND[a]	ND	ND	ND
2B4	ND	6 (7)	66 (73)	ND
2B5	1770 (590)	2850 (950)	ND	720 (240)

[a] ND, activity was not detected.

TABLE 2

Microsomal catalytic activities of HK 293-expressed P450 2B4 and 2B5

Substrate (benzphetamine, 1 mM; ethoxycoumarin, 0.3 mM; MDB, 1 mM; pentoxyresorufin, 10 μM; benzyloxyresorufin, 10 μM) was incubated with HK 293 microsomal preparations in the presence of NADPH or an NADPH-regenerating system. Each assay is described in Experimental Procedures. No exogenous P450 reductase was added to the microsomes. Each value is the average of duplicate determinations with pooled microsomes from each cell line. Turnover numbers were calculated based on the amount of enzyme expressed, determined using densitometry on a Western blot with purified P450 2B4 as standards. The values for substrate selectivity are based on the ratio of 2B4/2B5 turnover numbers.

Substrate	P450 2B4		P450 2B5		Substrate selectivity (2B4/2B5)
	pmol/min/mg	nmol/min/nmol	pmol/min/mg	nmol/min/nmol	
Benzphetamine	980	32.7	760	7.6	4.3
Ethoxycoumarin	115	3.5	25	0.25	14.0
MDB	155	5.5	33	0.35	15.7
Pentoxyresorufin	14	0.47	9	0.09	5.2
Benzyloxyresorufin	27	0.90	0.6	0.006	160

in COS-7 cells catalyzed benzyloxyresorufin *O*-dealkylation (BROD activity of 32, 57, and 25 pmol/min/mg, respectively), whereas this activity was not observed with the COS-expressed 2B5 form. These results in the expression systems demonstrate that BROD activity could be used as a selective marker for the P450 2B4, 2B-Bx, and 2B-B1 forms, without interference from P450 2B5.

Antibody inhibition of BROD activity in rabbit liver microsomes. Antibody inhibition experiments were performed with rabbit liver microsomes to determine whether the BROD activities observed with recombinant 2B4, 2B-Bx, and 2B-B1 forms were specific to enzymes in the P450 2B subfamily. Untreated or PB-induced microsomes were incubated with a single concentration of preimmune IgG or anti-P450 2B4 IgG (10 μg/μg of microsomal protein) before assessment of BROD activity. In untreated rabbit microsomes, BROD activity was inhibited by 89% when anti-2B4 IgG was added (Table 3). In PB-induced microsomes, >97% of the BROD activity was inhibited with anti-2B4 IgG. The large extent of inhibition of BROD activity by anti-2B4 IgG demonstrates that benzyloxyresorufin can also be used in both untreated and induced microsomes to monitor P450 2B4 activity, without interference from P450 forms from other subfamilies.

Inactivation of recombinant P450 2B4 and 2B5 by PCP. Several laboratories have studied the metabolism of PCP and the corresponding mechanism-based inactivation of P450s (23). In rabbits, the activities that were inhibited by PCP were those catalyzed by the P450 2B subfamily. Knowledge of the functional differences between P450 2B4 and P450 2B5 led us to investigate whether the two 2B forms could be differentially inactivated by PCP. Kinetic inactivation experiments were performed using the HK 293-expressed 2B4 and 2B5 forms (Fig. 5). At an initial PCP concentration of 0.1 mM, the recombinant 2B4 appeared to be selectively inactivated, compared with the 2B5 form. P450 2B4, measured in the HK 293 expression system using 7-ethoxycoumarin, exhibited biphasic inactivation, with a rate constant of 0.12 min^{-1} for the initial fast phase. The reason for this apparent biphasic time course is uncertain at this time. The expressed 2B5 form appeared to be inactivated at a slow but detectable rate ($k_i = 0.03\ min^{-1}$), using androstenedione 15α-hydroxylase activity as a marker.

PCP inactivation of P450 2B4 and 2B5 in PB-induced hepatic microsomes. PB-induced hepatic microsomes, which contained both P450 2B4 and 2B5 as judged by Northern blot analysis, were incubated with various concentrations of PCP to compare the inactivation of P450 2B4 and 2B5 in a more integrated system. Furthermore, specific marker activities al-

TABLE 3

Antibody inhibition of BROD activity in untreated and PB-treated rabbit liver microsomes

Microsomes (20 μg) were incubated at 37° for 2 min with 10 μM benzyloxyresorufin, 50 mM HEPES, pH 7.6, and 1 mM NADPH, in a final volume of 0.5 ml. The production of resorufin was determined fluorimetrically. Samples receiving antibody were incubated with 10 μg of IgG/μg of microsomal protein for 30 min at room temperature before addition of substrate, buffer, and NADPH. Incubations without IgG added were preincubated for 30 min at room temperature with an equivalent volume of PBS. The activity for each duplicate is shown.

Microsomes	Activity			Inhibition[a]
	No IgG	Preimmune IgG	Anti-2B4 IgG	
	nmol/min/mg			%
Untreated	0.25, 0.27	0.28, 0.25	0.03, 0.03	89.0
PB-treated	3.69, 3.65	3.21, 3.30	0.09, 0.08	97.4

[a] Difference in activity between microsomes incubated with preimmune IgG and anti-2B4 IgG.

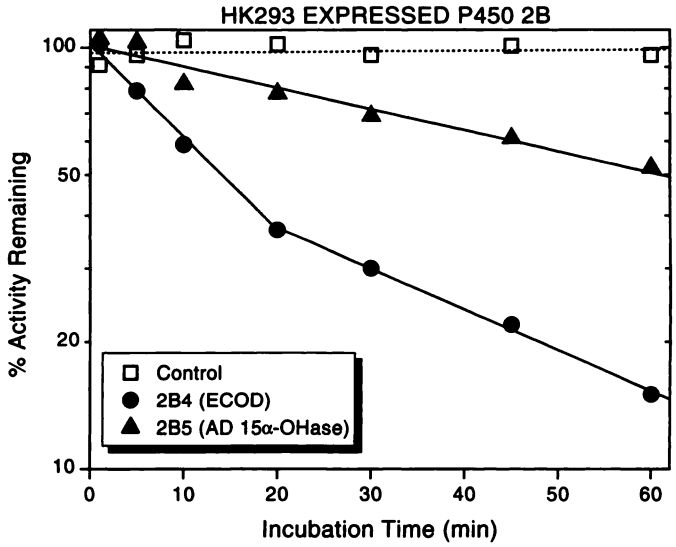

Fig. 5. Inactivation by PCP of P450 2B4 and 2B5 expressed in HK 293 cells. Incubations were performed with 0.1 mM PCP in microsomal fractions (5 mg/ml) from the HK 293 cell lines expressing recombinant 2B4 and 2B5. Aliquots were removed at various times to assay for ECOD (for expressed 2B4) or androstenedione (*AD*) 15α-hydroxylase (for expressed 2B5) activities.

lowed us to investigate the basis for the poor inactivation of P450 2B5 (Fig. 6). Benzyloxyresorufin was used in these experiments to monitor the inactivation of P450 2B4 and the other two, functionally related, P450 2B forms (P450 2B-B1 and 2B-Bx) that might be present in the microsomes. The androstenedione 15α-hydroxylase activity in the microsomes was used as a specific marker for P450 2B5. BROD was inactivated in the liver microsomes by PCP and exhibited biphasic kinetics at the higher concentrations, similar to those observed with recombinant P450 2B4. Inactivation of androstenedione 15α-hydroxylase displayed first-order kinetics, although little increase was observed in the inactivation rate constant for P450 2B5 over the concentration range. In addition to the low level of inactivation of 15α-hydroxylase activity, we noted that preincubation with PCP caused losses in other androstenedione hydroxylase activities (6β-hydroxylase and an unidentified activity) in PB-induced hepatic microsomes, an observation that suggests that PCP inactivates P450 forms from other subfamilies (data not shown). The observed rate constants were used to calculate apparent *KI* values for P450 2B4 and 2B5 in the microsomal system of 124 μM and 75 μM, respectively. The maximal rate constant for inactivation of P450 2B5 was 3-fold lower than for 2B4 (0.05 min^{-1} versus 0.17 min^{-1}, respectively). Therefore, the poor ability of PCP to inactivate P450 2B5 is a result of a low maximal rate constant. This inference was confirmed with HK 293-expressed P450 2B4 and 2B5, in that >4-fold differences in the rate constants remained at 1 mM PCP (data not shown). In addition to evaluation of PCP as a mechanism-based inactivator, these experiments also demonstrate the utility of BROD and androstenedione 15α-hydroxylase activities to monitor P450 2B4 and 2B5 activity in microsomes.

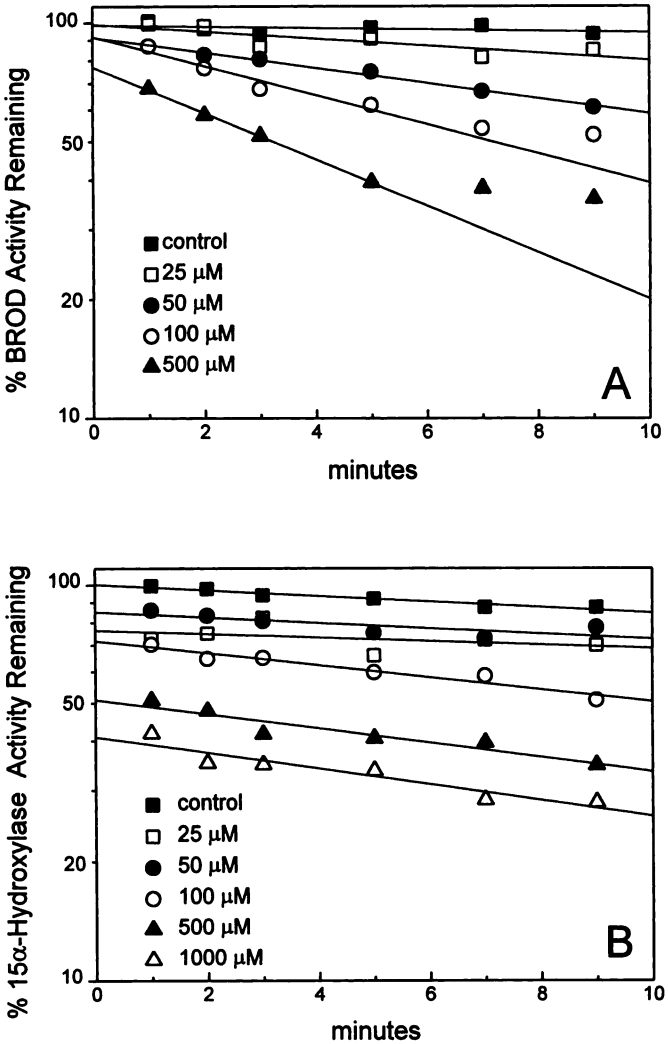

Fig. 6. Time- and concentration-dependent inactivation by PCP of P450 2B4 and 2B5 activities in PB-treated liver microsomes. Hepatic microsomes from PB-treated rabbits were incubated with the indicated concentrations of PCP. Residual BROD (A) and androstenedione 15α-hydroxylase (B) activities were measured at various times during the inhibitor preincubation, as described in Experimental Procedures. The *lines* shown were drawn by linear regression analysis of the natural logarithm of the percentage residual activity as a function of inhibitor incubation time up to 5 min. The 100% values for BROD and androstenedione 15α-hydroxylase activities for the microsomal samples used in the inactivation experiments were 2.9 and 0.45 nmol/min/mg of protein, respectively.

Discussion

Our previous investigations with a small number of substrates demonstrated that three of the four known rabbit P450 2B enzymes (P450 2B4, 2B-B1, and 2B-Bx) are functionally indistinguishable, whereas P450 2B5 is catalytically unique. In this investigation, Northern hybridization experiments showed that four of 10 PB-treated animals were totally deficient in hepatic P450 2B5 mRNA. The regio- and stereospecific pattern of androstenedione hydroxylation in hepatic microsomes from individual PB-treated rabbits was shown to be dramatically different in those animals that were devoid of P450 2B5. Stable expression in HK 293 cells has now allowed us to characterize the substrate selectivities more fully and to investigate the mechanism-based inactivation of P450 2B5 and P450 2B4 (as a representative of the three functionally similar P450 2B forms). PCP was found, using the recombinant enzymes, to be a selective mechanism-based inactivator of P450 2B4 versus 2B5. In the process of evaluating four new substrates, BROD was identified as an activity of P450 2B4 but not P450 2B5. The poor inactivation of P450 2B5 by PCP was found, using the 2B4- and 2B5-selective markers in hepatic microsomes, to result from a low maximal rate constant.

Androstenedione 15α-hydroxylase activity in PB-induced livers from individual rabbits was significantly correlated with the level of 2B5 mRNA on Northern blots. Androstenedione 15α-hydroxylase activity should be a valuable specific marker in future studies of rabbit P450 2B5,[3] whereas P450 forms from other subfamilies contribute to the 6β- and 16α-hydroxylase activities observed in microsomes. The profound alteration in the regio- and stereoselectivity of androstenedione hydroxylation in the P450 2B5-null phenotype suggests that variations in the expression of functionally different P450 2B forms between individual animals may be an important consideration in studies of the effects of PB-type induction on xenobiotic and androgen metabolism.

The substrates evaluated in this investigation are commonly used to measure P450 2B induction or to monitor the activity of microsomal or purified P450 2B4 during enzyme inactivation or other mechanistic investigations. Using the heterologously expressed enzymes, benzyloxyresorufin metabolism was found to be specific to P450 2B4 and its related forms (2B-Bx and

2B-B1).[4] BROD was nearly completely inhibited by anti-P450 2B4 IgG in both untreated and PB-induced liver microsomes. The antibody inhibition results, together with the heterologous expression studies, confirm that BROD activity can be used as a specific marker of P450 2B4 activity in microsomes, without contributions to this activity from P450 2B5 or P450 forms from other subfamilies. In reconstituted systems, the alkoxyresorufin substrates are rapidly reduced by exogenously added NADPH-P450 reductase (35).[5] Therefore, benzyloxyresorufin is not a reliable marker for monitoring the activity of purified reconstituted 2B4. Interestingly though, the discovery of benzyloxyresorufin metabolism as a specific activity of P450 2B4 might not have been possible using other, nonmammalian, expression systems that require the addition of exogenous reductase.

Whereas benzyloxyresorufin dealkylation was specific for the 2B4-related forms, the metabolism of the other substrates studied here (benzphetamine, ethoxycoumarin, pentoxyresorufin, and MDB) was catalyzed to some extent by both P450 2B4 and 2B5. The catalytic activities of P450 2B4 towards these substrates were 4–16-fold higher than those measured for P450 2B5 in the HK 293 expression system. Benzphetamine and ethoxycoumarin have been used previously as catalytic markers for P450 2B4 function in lung and PB-induced liver microsomes (17) and may continue to be useful markers of P450 2B4 activity in rabbit lung and kidney microsomes, where the 2B5 form is not expressed (6, 13). For hepatic microsomes, the compounds may not be optimal P450 2B4 markers, because the measured activities depend to some extent on whether the donor animal expresses the P450 2B5 enzyme.

The turnover numbers for P450 2B4-catalyzed metabolism of benzphetamine and MDB in the HK 293 expression system appear to be 2–4-fold lower than the values reported for purified reconstituted P450 2B, whereas the ECOD activity for HK 293-expressed 2B4 is similar to that reported (18, 21, 23). Differences between the activities observed in heterologous expression systems and in reconstituted systems can have several explanations, including the expression of nonfunctional enzyme or differences in the ratio of P450 to NADPH-P450 reductase. Purified "P450 2B4" preparations may contain mixtures of two or three P450 2B forms, with each form contributing to the overall activity of the preparation. Because investigators have not yet purified P450 2B forms from phenotyped animals, the possibility of multiple P450 2B forms in purified preparations precludes completely reliable comparisons of the metabolic activities of a single heterologously expressed form and purified P450 2B enzyme.

The metabolism of PCP has been the subject of investigation in several laboratories, because of the ability of PCP to cause the covalent binding and mechanism-based inactivation of P450 (23, 36–38). Liver microsomes from PB-treated rabbits were shown to metabolize PCP, and purified P450 2B4 was found be subject to the metabolism-dependent inactivation caused by PCP. However, the presence of P450 2B5 in the purified preparation was not determined in those investigations. In addition, previous studies used less selective substrates, such as benzphetamine and 7-ethoxycoumarin, to monitor the inactivation of P450 2B4 in rabbit liver microsomes and reconstituted systems. Using a stable heterologous expression system, we established that PCP selectively inactivates 2B4, with little effect on P450 2B5. The selectivity of the mechanism-based inactivation by PCP further exemplifies the functional dissimilarity between these two P450 enzymes, which differ at only 11 of 491 amino acid residues. To show the utility of microsomal markers for P450 2B4 and 2B5 and to determine the basis for poor inactivation of P450 2B5, we used BROD and androstenedione 15α-hydroxylase assays to evaluate the kinetics of inactivation of P450 2B4 and 2B5, respectively, in microsomes. Even though the apparent K_I values were not different, the maximal rate constant for inactivation of androstenedione 15α-hydroxylase was much lower than that for BROD in microsomes. Poor inactivation of P450 2B5 may be related to several steps in the inactivation process, including the rate of PCP conversion to reactive species and the partition ratio, i.e., the number of turnovers per inactivation event. Selective inactivation by PCP will be useful in the future for investigating structure-function relationships for P450 2B4 and 2B5. Changes in the kinetics of inactivation have been used recently, in conjunction with site-directed mutagenesis, to help pinpoint amino acid residues in P450 2B1 that are critical for its catalytic function (11, 33, 34).

HK 293 cells represent a novel system for the expression of P450 enzymes in a stable mammalian cell line. These cells have been used previously to express pharmacologically important proteins such as cGMP-gated ion channels (39). The stable nature of this mammalian expression system is an advantage over labor-intensive transient expression systems (such as COS cells) when a source of a single P450 form is required over an extended period of time. HK 293 cells provided a continuous source of P450 2B4 and 2B5 at sufficient levels to allow us to conduct studies on their substrate specificities and mechanism-based inactivation. Also, enough NADPH-P450 reductase is present in these cells to obviate the requirement for addition of exogenous flavoprotein to microsomal incubations, a step that frequently occurs with the use of nonmammalian expression systems. As with other mammalian cell lines used to express P450s, HK 293 cells might be useful for assessing cytotoxicity due to xenobiotic bioactivation by a particular P450 form.

In conclusion, the polymorphic expression of P450 2B5 can profoundly influence the metabolic disposition of androstenedione and, therefore, may similarly modify the biotransformation of some xenobiotic substrates. The changes in metabolic disposition and any toxic or pharmacological consequences associated with the induction of P450 2B enzymes would be determined by interanimal differences and tissue specificity in the expression of highly related forms, as well as the structural features of the substrate. In rabbits, the use of BROD and androstenedione 15α-hydroxylation as specific markers for P450 2B4 and 2B5 activity, respectively, will now allow more specific measurement of P450 2B4 and 2B5 in liver microsomes, where both forms may be expressed concomitantly. For future investigations of structure-function relationships for P450 2B enzymes, we would like to identify selective mechanism-based

[4] The 2B-Bx and 2B-B1 forms differ from 2B4 at four and six amino acid residues, respectively (13). These amino acid differences fall outside of the substrate recognition sites that have been reported for P450 2B forms and, therefore, would not be expected to cause changes in substrate specificity. However, several of the 11 differences between 2B4 and 2B5 fall within substrate recognition sites and align with residues that are important for determining substrate specificity in rat 2B1 (33, 34).

[5] S.W. Grimm and J.R. Halpert, unpublished observations.

inactivators of the 2B5 form, as we have here with PCP for P450 2B4.

Acknowledgments

The authors are grateful to Michael McLane and Tyrell Norris, ZENECA Biomedical Research, for the synthesis of the oligonucleotides and also for helpful discussions and advice on the expression of proteins in HK 293 cells. We also thank Karen R. Stams for her assistance in maintaining the stable cell lines. The skill of James Huffmaster in preparing the figures in this manuscript is also greatly appreciated.

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