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Species-Dependent Expression and Induction of Homologues of Rabbit Cytochrome P-450 Isozyme 5 in Liver and Lung

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SUMMARY

The presence of homologues of rabbit cytochrome P-450 isozyme 5 in pulmonary and hepatic microsomal preparations from guinea pig, mouse, monkey, hamster, and rat was examined by immunoblotting and inhibition of metabolism of 2-aminofluorene with antibodies to isozyme 5. Homologues to isozyme 5 were detected in pulmonary preparations from all five species. However, only hepatic preparations from hamster, in addition to those from rabbit, contained detectable levels of this isozyme. With the exception of induction by phenobarbital in rabbit liver, treatment of animals with phenobarbital or tetrachlorodibenzo-*p*-dioxin did not increase hepatic or pulmonary content of isozyme 5 homologues or the amount of 2-aminofluorene metabolism inhibited by antibodies to isozyme 5. Metabolism of 2-aminofluorene was measured both colorimetrically (formation of a reduced iron chelate from the *N*-hydroxyfluorene metabolite) and radiochemically (separation of ^{3}H-metabolites by high performance liquid chromatography and quantitation by scintillation counting). A turnover number of 48 nmol of product × min^{-1} × nmol of enzyme^{-1} for isozyme 5-catalyzed metabolism of 2-aminofluorene was determined with incubations containing isozyme 5 purified from rabbit lung. A similar turnover number was calculated from the rabbit hepatic microsomal activity inhibited by antibodies to isozyme 5 and the microsomal isozyme 5 content measured by immunoquantitation. In other species, amounts of metabolism inhibited by antibodies to isozyme 5 agreed qualitatively with relative staining intensities on immunoblots. In all species except the hamster, rates of total and isozyme 5-catalyzed metabolism of 2-aminofluorene were greater with pulmonary than with hepatic microsomal preparations from untreated animals.

Metabolism by the P-450 system initiates the process by which many exogenous compounds are excreted by humans and other animals. However, the P-450 system also converts some compounds into highly reactive products that initiate carcinogenic, mutagenic, or other toxic responses by binding covalently to cellular macromolecules. Aromatic amines, and amides, like a number of other classes of compounds, undergo both types of reactions: detoxification, resulting from ring-hydroxylation, and activation, resulting from N-hydroxylation (1, 2).

Hepatic preparations from rabbit, rat, mouse, hamster, and guinea pig metabolize the aromatic amide, AAF, to products that exhibit mutagenic activity (1, 3). This activity has been ascribed to N-hydroxylation catalyzed by an isozyme of cytochrome P-450 that is induced by treatment *in vivo* with 3-methylcholanthrene (1). With preparations from rabbit, however, a significant proportion of the microsomal metabolism of AAF to mutagenic products is initiated by deacetylation to AF followed by N-hydroxylation catalyzed by isozyme 5, a form of cytochrome P-450 induced in liver by treatment of rabbits with PB (4). The metabolism of both AAF and AF to mutagenic products in rabbit pulmonary microsomal preparations appears to be mediated entirely by isozyme 5 (3) as is the N-hydroxylation of AF in preparations from rabbit urinary bladder (5). Isozyme 4, which is active in the metabolism of aromatic amines and amides to mutagenic products and is induced in liver by 3-methylcholanthrene, is not detected in rabbit lung (6) or bladder (5).

The known structural, immunochemical, and catalytic properties of isozyme 5 show that it is a distinct form of cytochrome P-450 present in rabbit liver and extrahepatic tissues (5). None of the forms of cytochrome P-450 isolated from rat or other species appear to have properties similar to those of isozyme 5. Although PB induces the synthesis of both isozyme 5 and isozyme 2 in rabbit liver (4), the relationship between these isozymes is not comparable to that between P-450$_b$ and P-450$_e$, highly related isozymes that are induced by PB in rat liver (7).

In the present study, we have used immunochemical methods and activity determinations to identify homologues of rabbit isozyme 5 in rat, mouse, hamster, guinea pig, and monkey. The

ABBREVIATIONS: P-450 system, cytochrome P-450 monooxygenase system; AAF, 2-acetylaminofluorene; AF, 2-aminofluorene; PB, phenobarbital; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; HPLC, high performance liquid chromatography; α-NF, α-naphthoflavone; TPTZ, 2,4,6-tripyridyl-*S*-triazine; anti-2 and anti-5, anti-isozymes 2 and 5, respectively.

participation of this isozyme in the pulmonary and hepatic microsomal metabolism of AF is described.

Materials and Methods

Animals and treatments. Fischer rats (8 weeks), Hartley guinea pigs (8–10 weeks), and Syrian hamsters (13 weeks) were obtained from Charles River Breeding Laboratories (Wilmington, MA); C57BL mice (10 weeks) were from The Jackson Laboratory (Bar Harbor, ME); and rabbits (25 weeks) were from Dutchland Farms (Denver, PA). All animals were males. Sodium PB in phosphate-buffered saline was administered intraperitoneally daily for 4 days (40 mg/kg for rabbits, 80 mg/kg for other species). Animals were starved for 1 day before sacrifice. TCDD in corn oil was administered intraperitoneally (20 μg/kg for rats, mice, hamsters, and guinea pigs; 10 μg/kg for rabbits) 96 hr before killing.

Preparation of microsomal fractions. Pulmonary and hepatic microsomal preparations were made from tissue pools of 10 rats, 40 mice, 15 hamsters, 5 guinea pigs, or individual rabbits. Microsomes were prepared by standard differential centrifugation procedures (8).

Metabolism of AF. N-Hydroxylation of AF was determined colorimetrically by the method of Belanger *et al.* (9) with some modifications. AF (50 μM), microsomal protein (20–100 μg/ml), NADPH (2 mM), and potassium phosphate buffer (100 mM, pH 7.4) were mixed in a volume of 2 ml and incubated for 10 min at 37°. (Concentrations of AF >50 μM interfered with the assay.) Reactions were stopped by adding 0.5 ml of the incubation mixture to 2.5 ml of colorimetric reagent: sodium acetate (5 M, pH 5.9), TPTZ (0.24 M), and $FeCl_3$ (80 μM). Absorbance was read at 595 nm after 30–60 min of color development at room temperature.

Radiochemical determination of AF metabolism employed an HPLC procedure described previously (5). Incubations contained microsomal protein (200–1000 μg/ml), ^{3}H-AF (250 μM), and NADPH (2 mM) made up to 1 ml with potassium phosphate buffer (100 mM, pH 7.4). After incubating 10 min at 37°, reactions were stopped by the addition of ice-cold ethyl acetate (twice in 4 ml). Extracts were concentrated by N_2 stream and residues were dissolved in 100 μl of methanol. Metabolites were separated by reverse phase HPLC on a C-18 μBondapak column (Waters Associates, Milford, MA) using a fully automated Waters system (600a pumps, system controller, and WISP). Metabolites were eluted with a gradient from 80% water/20% methanol to 100% methanol and identified as previously described (5). Quantitation was by scintillation counting (Flo-One IC, Radiomatic Instruments). Columns were treated with 0.2% acetohydroxamic acid in the solvents (10).

Metabolism in reconstituted systems. Purified cytochrome P-450 and NADPH-cytochrome P-450 reductase were combined for assays of AF metabolism as previously described (3). First, lipid (25 μg of phosphatidylcholine) was allowed to dry on the bottom of a conical tube. Purified isozyme 5 (7.5–50 pmol) and NADPH-cytochrome P-450 reductase were then added and the mixture was incubated on ice for 15 min. AF, buffer, and NADPH were added as described (3).

Electrophoresis and immunochemical procedures. Electrophoresis on polyacrylamide gels (6%) in the presence of sodium dodecyl sulfate (11), immunoblotting (12), and immunochemical staining (13) were done by standard procedures. The antibodies to cytochrome P-450 isozymes 2 and 5 were those described previously (14).

Other analytical procedures. Cytochrome P-450 concentrations were determined by the methods of Omura and Sato (15) or Estabrook *et al.* (16). Protein was determined by the method of Lowry *et al.* (17).

Chemicals. Acetohydroxamic acid, NADPH, and TPTZ were purchased from Sigma Chemical Co. (St. Louis, MO). ^{3}H-AF was purchased from Midwest Research Institute (Kansas City, MO). AF and α-NF were purchased from Aldrich (Milwaukee, WI). Immunochemical reagents were purchased from Cappel (Cooper Biomedical, Malvern, PA), electrophoresis chemicals were from Hoefer Instruments (San Francisco, CA), and nitrocellulose paper was from Schleicher and Schuell (Keene, NH).

Results

Detection of isozyme 5 homologues in pulmonary microsomal preparations from several species. Homologues of isozyme 5 were detected on immunoblots of pulmonary microsomal preparations from rat, hamster, monkey, mouse, and guinea pig (Fig. 1). The order of staining intensities with samples containing 20 μg of microsomal protein was: guinea pig > mouse > monkey = hamster > rat. The staining intensity with 5 μg of microsomal protein from rabbit was 2 to 3 times that with 20 μg of protein from guinea pig. (Results with samples containing greater than 20 μg of protein are shown in the following figures.)

With pulmonary microsomes from rabbit, isozyme 5 is easily distinguished from the other major cytochrome P-450 present in lung, isozyme 2, by a large difference in mobilities and little or no immunochemical cross-reactivity (4, 5, 14). In contrast, we were unable to determine by the standard immunoblotting method whether the proteins stained by anti-2 and anti-5 in pulmonary microsomal preparations from other species were actually different. Therefore, a modified procedure was developed so that the relative mobilities and immunochemical reactivities of these proteins could be accurately determined. Immunoblots of pulmonary and hepatic microsomal samples were divided so that half of each sample was stained with anti-5 and the other half with anti-2. The blots were then aligned and

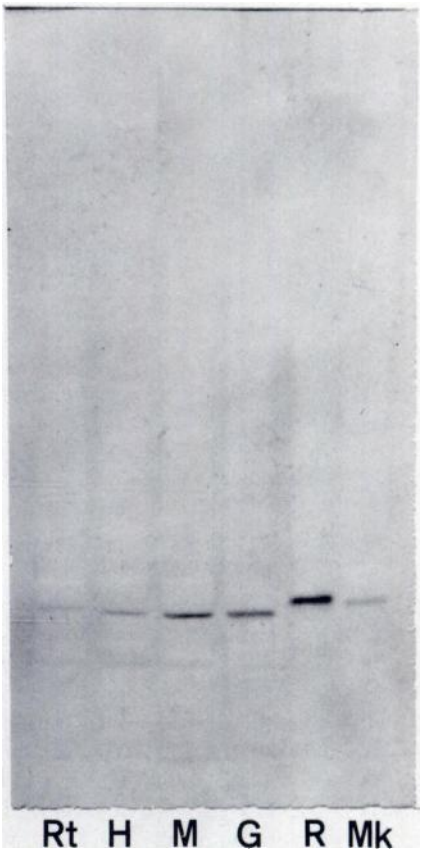

Fig. 1. Detection of proteins by anti-5 on immunoblots of pulmonary microsomal preparations from animals treated with PB. Samples from rat (*Rt*), hamster (*H*), mouse (*M*), guinea pig (*G*), and monkey (*Mk*) contained 20 μg of microsomal protein; the sample from rabbit (*R*) contained 5 μg. Procedures for electrophoresis, immunoblotting, and staining are listed under Materials and Methods.

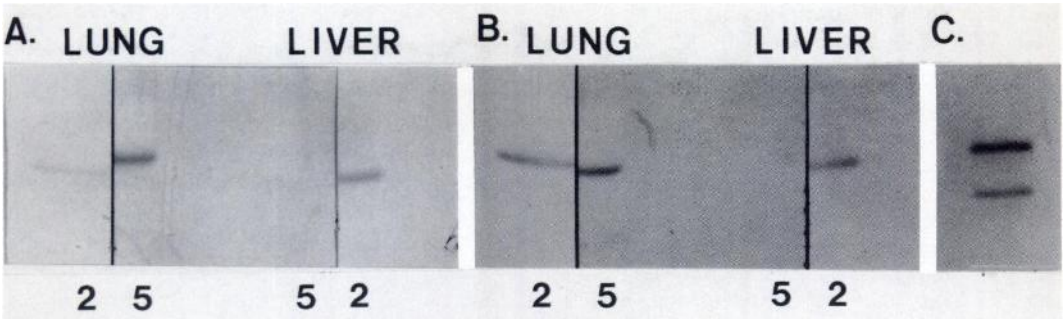

Fig. 2. Detection of proteins by anti-5 and anti-2 on immunoblots of pulmonary and hepatic microsomal preparations from guinea pig (A) and mouse (B), and pulmonary microsomal preparations from rabbit (C). Pulmonary (100 μg) and hepatic (10 μg) microsomal samples from guinea pig and mouse were applied to polyacrylamide gels in 15-mm wells. Following electrophoresis and transfer, the nitrocellulose sheets were cut from top to bottom so that half of each sample could be stained with either anti-5 (*5*) or anti-2 (*2*). The stained sheets were then realigned as shown. Rabbit pulmonary microsomal proteins (5 μg) were stained with a mixture of anti-5 and anti-2.

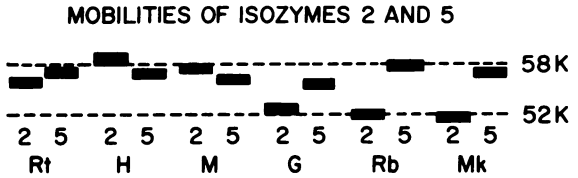

Fig. 3. The relative mobilities of isozymes 2 (*2*) and 5 (*5*) and their homologues as determined by immunoblotting of pulmonary microsomal preparations from rat (*Rt*), hamster (*H*), mouse (*M*), guinea pig (*G*), rabbit (*Rb*), and monkey (*Mk*).

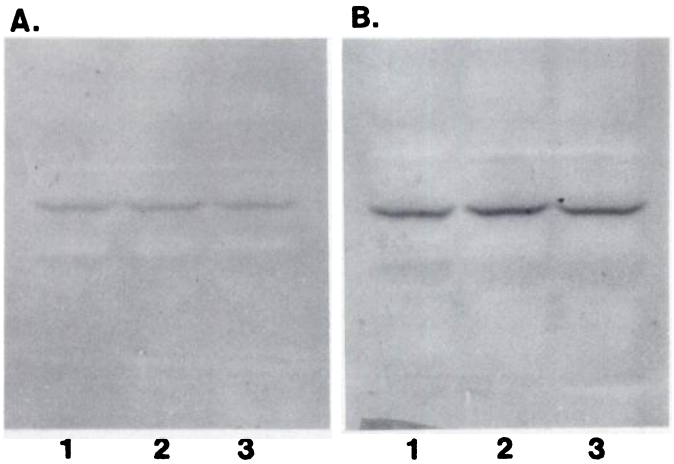

Fig. 4. Effects of TCDD and PB on the concentrations of proteins detected by anti-5 in pulmonary microsomal preparations from hamsters (A) and rats (B). Samples of pulmonary microsomal preparations (100 μg of protein) from untreated (*lane 1*), PB-treated (*lane 2*), and TCDD-treated (*lane 3*) hamsters and rats were examined.

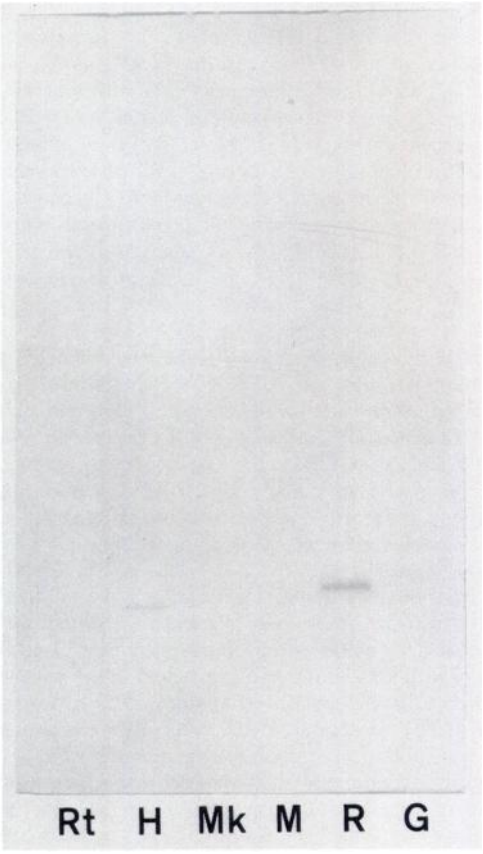

Fig. 5. Detection of proteins by anti-5 on immunoblots of hepatic microsomal preparations from animals treated with PB. Samples from rat (*Rt*), hamster (*H*), monkey (*Mk*), mouse (*M*), and guinea pig (*G*) contained 30 μg of protein; the sample from rabbit (*R*) contained 5 μg of protein.

examined. Results obtained with samples from guinea pigs and mice are shown in Fig. 2, A and B. With this method it was clear that the mobilities of the pulmonary proteins stained by anti-2 and anti-5 were distinctly different. In addition, the failure of anti-5 to react with the hepatic samples demonstrated further that the pulmonary results were not due to cross-reactivity of anti-5 with isozyme 2 homologues. Small, but distinct, differences between the mobilities of isozyme 2 and 5 homologues were also observed with pulmonary microsomal preparations from rats, hamsters, and monkeys (not shown). The relative mobilities of the isozyme 2 and 5 homologues vary with species: the mobilities of isozyme 2 and its homologues in rabbit, rat, guinea pig, and monkey are greater than those of isozyme 5 and its homologues; the opposite is true with the hamster and mouse (Fig. 3).

PB and TCDD, which are known to induce the synthesis of a number of isozymes of cytochrome P-450 in liver, were found to have no obvious effect on the pulmonary concentration of homologues of isozyme 5. Results obtained with hamsters and rats are shown in Fig. 4; similar results were obtained with mice and guinea pigs.

Detection of homologues of isozyme 5 in hepatic microsomal preparations. Homologues to isozyme 5 were de-

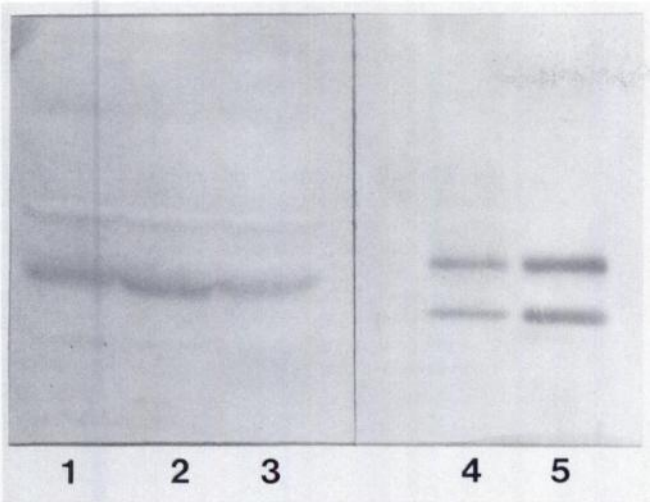

Fig. 6. Effects of PB and TCDD on the concentration of the protein detected by anti-5 in hepatic microsomal preparations from the hamster. Samples of hepatic microsomal preparations (100 μg of protein) from untreated (*lane 1*), PB-treated (*lane 2*), and TCDD-treated (*lane 3*) hamsters were examined. For comparison, samples of hepatic microsomal preparations from untreated (*lane 4*, 5 μg) and PB-treated (*lane 5*, 2 μg) rabbits, stained with a mixture of anti-5 (*upper band*) and anti-2 (*lower band*), are shown.

tected in hepatic microsomal preparations from only two of the species tested, hamster and rabbit (Fig. 5). This was the case with samples of up to 100 μg of protein from both untreated and PB-treated animals. In contrast to its effect in rabbits, treatment of hamsters with PB did not appear to alter the hepatic content of the isozyme 5 homologue (Fig. 6). Results with TCDD suggest that it may cause a slight decrease in the content of the isozyme 5 homologue in hamster liver (Fig. 6).

Metabolism of AF in pulmonary and hepatic microsomal preparations. Hepatic and pulmonary microsomal preparations from all species tested were active in the metabolism of AF (Fig. 7). Colorimetric and HPLC techniques gave similar, but not identical, results. These results indicate that the colorimetric assay, which is significantly more sensitive than the HPLC assay, can be used to qualitatively assess the N-hydroxylation of AF. Metabolism of AF varied over a 10-fold range among species. The highest pulmonary activities were found with rabbit and guinea pig; lower activities were found with mouse followed by rat and hamster. Except for the hamster, pulmonary activities were greater than hepatic activities in preparations from untreated animals. Anti-5 inhibited the pulmonary microsomal metabolism of AF by 70% or more in preparations from rabbits, rats, guinea pigs, mice, and hamsters; similar results were obtained with either assay (Fig. 8). In contrast, inhibition of hepatic activity by anti-5 was less than 15% except for 40–60% inhibition observed with preparations from hamsters and rabbits (Fig. 8). The large tissue and species differences in isozyme 5-catalyzed metabolism of AF are readily apparent when comparisons of amounts of activity inhibited by anti-5 are made (Fig. 9). Fig. 9 also shows that treatment of animals with PB increases isozyme 5-catalyzed hepatic metabolism only in the case of the rabbit. This was also true for total metabolism of AF (not shown).

Metabolism of AF catalyzed by isozymes 4 and 5 in rabbit hepatic microsomal preparations. Concentrations of isozymes 4 and 5, rates of metabolism of AF, and inhibitory effects of anti-5, anti-reductase, and α-NF (an inhibitor of isozyme 4) in hepatic microsomal preparations from untreated, PB-treated, and TCDD-treated rabbits were determined (Table 1). Treatment of rabbits with PB increased the concentration of isozyme 5 and the metabolism of AF; treatment with TCDD increased the concentration of isozyme 4 but had little effect on the metabolism of AF. Anti-reductase inhibited the metabolism of AF by greater than 80% in all three preparations, whereas anti-5 inhibited the reaction by greater than 80% only in preparations from untreated and PB-treated animals. In microsomal preparations from rabbits treated with TCDD, 42% inhibition was obtained with anti-5 and 53% with α-NF. In preparations from untreated and PB-treated animals, inhibition by α-NF was less than 5%. The specific activities of isozyme 5 with AF, calculated from the concentrations of isozyme 5, and the activities inhibited by anti-5, were: 52 (untreated), 41 (TCDD-treated), and 45 (PB-treated) nmol of product × min^{-1} × nmol of isozyme 5^{-1}. The specific activity of isozyme 4 was 0.9 nmol of product × min^{-1} × nmol of isozyme 4^{-1} in preparations from TCDD-treated animals. Based on this value, only 7% of the activity observed with the preparations from untreated rabbits and 4% of the activity of those

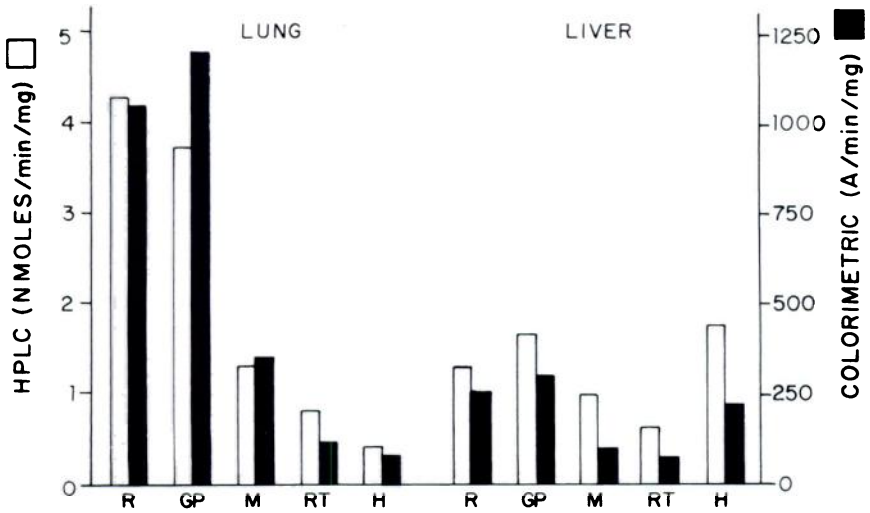

Fig. 7. Metabolism of AF in pulmonary and hepatic microsomal preparations: comparison of HPLC and colorimetric techniques. Total metabolic products (nmol/min/mg) were measured by the HPLC technique (□), and absorbance of the reduced iron-TPTZ chelate (*A/min/mg*) was measured by the colorimetric technique (■). Preparations from rabbits (*R*), guinea pigs (*GP*), mice (*M*), rats (*RT*), and hamsters (*H*) were examined. The results shown are the average of not less than two determinations for each of two separate preparations from liver and lung.

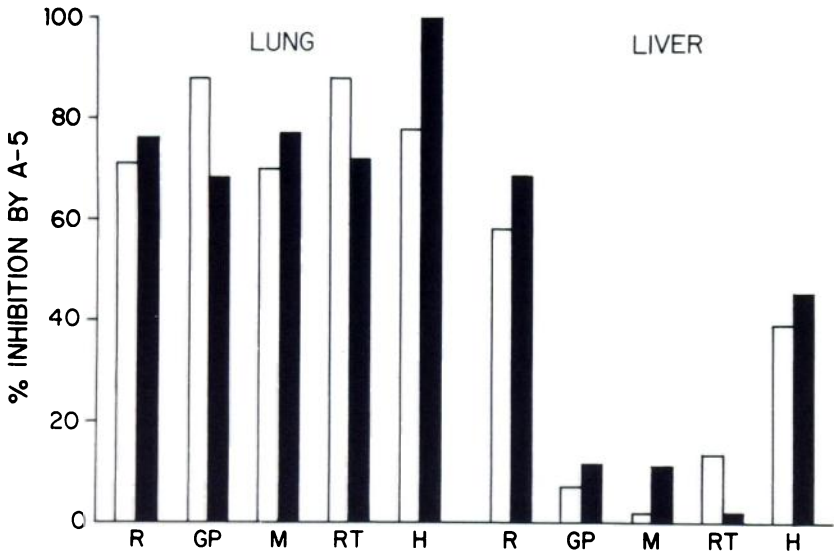

Fig. 8. Per cent inhibition by anti-5 of pulmonary and hepatic microsomal metabolism of AF. The effect of anti-5 (*A-5*) on the metabolism of AF, as determined by the HPLC (□) or colorimetric (■) methods, was determined with preparations from untreated rabbits (*R*), guinea pigs (*GP*), mice (*M*), rats (*RT*), and hamsters (*H*). The results shown are the average of not less than two determinations for each of two separate preparations from liver and lung.

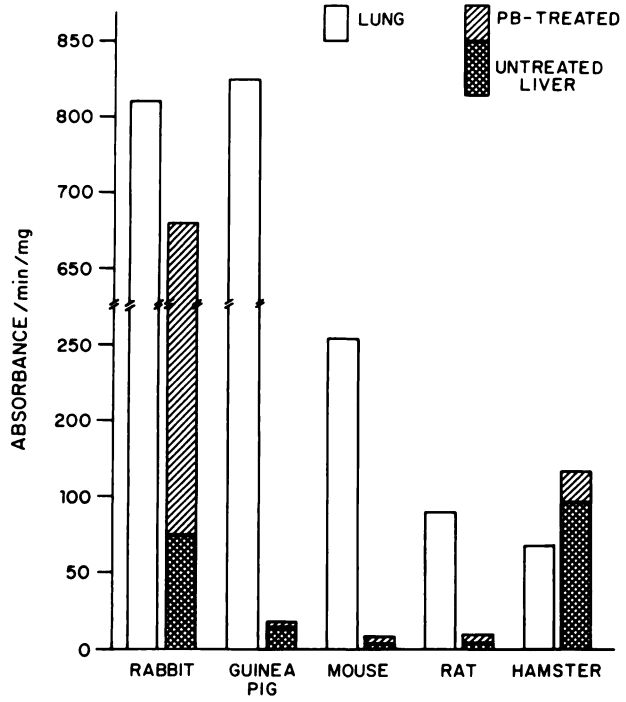

Fig. 9. Relative rates of AF metabolism catalyzed by isozyme 5 homologues in pulmonary microsomal preparations from untreated animals and in hepatic microsomal preparations from untreated and PB-treated animals. The amounts of activity inhibited by anti-5, as determined by the colorimetric assay (absorbance/min/mg), are reported for pulmonary preparations, and hepatic preparations from untreated and PB-treated animals, as indicated by the key (*top*).

from PB-treated rabbits would have been catalyzed by isozyme 4.

Metabolism of AF by purified isozyme 5. The metabolism of AF catalyzed by purified isozyme 5 in reconstituted systems was analyzed by both the HPLC and colorimetric methods. The elution profile seen with HPLC showed the same five metabolites (ring-hydroxylated AF, N-hydroxylated AF, nitrosofluorene, unknown, and azoxyfluorene) described previously for microsomal incubations (18). The rate of metabolism of AF in the reconstituted system was 47.9 ± 9.6 nmol of product × min^{-1} × nmol of isozyme 5^{-1} ($n = 6$), a value similar

TABLE 1

Metabolism of AF by hepatic microsomal preparations from untreated, PB-treated, and TCDD-treated rabbits

Microsomal preparation	Rate[a]	Inhibition[b]			Isozyme content[c]		Turnover number[d]	
		a-R	a-5	α-NF	5	4	5	4
				%	*nmol/mg protein*			
Untreated	1.3	89	88	<5	0.022	0.104	52	
TCDD	1.5	81	42	53	0.016	0.933	41	0.9
PB	4.8	84	82	<5	0.085	0.240	45	

[a] The rate is nmol of product × min^{-1} × mg of $protein^{-1}$.
[b] Inhibition by antibodies to reductase (a-R), antibodies to isozyme 5 (a-5), and α-NF was determined.
[c] Isozyme contents were determined by immunoblotting.
[d] Turnover numbers for isozymes 4 and 5 were calculated by dividing the amount of activity inhibited (inhibition by α-NF for isozyme 4 and inhibition by anti-5 for isozyme 5) by the isozyme content.

to those calculated for isozyme 5-catalyzed metabolism of AF in microsomal incubations. No metabolism was observed in the absence of isozyme 5, and metabolism catalyzed by isozyme 5 was not inhibited by α-NF.

Discussion

Cytochrome P-450 isozyme 5 is a major component of the rabbit pulmonary P-450 system (19), is present in rabbit liver (4, 20), and is induced in liver by treatment of rabbits with PB (4, 20). We have now detected homologues of isozyme 5 in pulmonary microsomal preparations from rat, mouse, hamster, guinea pig, and monkey. Identification of these homologues by their reactivities with anti-5 on immunoblots is confirmed by inhibition of pulmonary microsomal metabolism of AF by anti-5. As is the case with rabbits, the pulmonary concentrations of these isozymes are not altered by treatment *in vivo* with PB or TCDD. In contrast to the consistent results obtained with pulmonary preparations, marked species differences are observed with hepatic preparations; only two species, rabbit and hamster, contain levels of hepatic microsomal protein that can be detected by anti-5 on immunoblots.[1] These findings are also

[1] Based on the staining intensities obtained with pulmonary microsomal preparations, the levels of isozyme 5 homologues in livers would be <2% of the pulmonary levels for mice and guinea pigs and <20% for rats.

confirmed by the effects of anti-5 on the metabolism of AF: marked inhibition of hamster and rabbit hepatic activities, and little or no inhibition of mouse, guinea pig, or rat activities.

With respect to homologues of isozyme 5 in liver, a second major difference among species is evident when hepatic microsomal preparations from animals treated with PB are examined. Treatment of rabbits with PB results in substantial increases in isozyme 5 content and isozyme 5-catalyzed activity (4, 20). However, PB has no obvious effect on the level of the isozyme 5 homologue in hamster liver, nor does it result in the synthesis of detectable levels of isozyme 5 homologues in livers of other species. In contrast, hepatic concentrations of isozyme 2 homologues are increased by PB in all of these species (21).

Our results show that there can be marked tissue and species differences in the isozymes of cytochrome P-450 that metabolize a given substrate. In rabbit liver, isozyme 5 plays a major role in the N-hydroxylation of AF, although it is clear that isozyme 4 is involved, particularly in animals treated with TCDD. This also appears to be the case in hamster liver. In livers of other species the initial association of aromatic amine N-hydroxylation with homologues of isozyme 4 (1) may be valid. However, this is clearly not the case for pulmonary metabolism. In addition to the evidence that pulmonary metabolism of AF is catalyzed by isozyme 5 homologues, homologues of isozyme 4 have not been detected in lungs of any species (6, 21, 22). Therefore, it appears that the majority, if not all, of the N-hydroxylation of AF in liver and lung of rats, mice, and guinea pigs is catalyzed by distinctly different isozymes of cytochrome P-450. The lack of isozyme 5 homologues in livers of rats and mice also points to a potential shortcoming in the *Salmonella* mutagenicity assay (Ames test) in which hepatic preparations from these species are generally employed (23).

In extending our studies of rabbit cytochrome P-450 isozyme 5 to other species, we have discovered a uniformity among pulmonary P-450 systems that is not duplicated in the liver. Further investigation of these tissue and species differences should provide some clues to the factors that control the expression of cytochrome P-450 isozymes.

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