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Participation of a Rat Liver Cytochrome P-450 Induced by Pregnenolone 16α-Carbonitrile and Other Compounds in the 4-Hydroxylation of Mephenytoin

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SUMMARY

Mephenytoin 4-hydroxylation, which has been found to be one of the reactions showing genetic polymorphism in humans, has been studied using rat liver microsomes. Pregnenolone 16α-carbonitrile, dexamethasone, troleandomycin, and phenobarbital (but not β-naphthoflavone) induced the hydroxylation activity to various extents. Mephenytoin itself also increased 4-hydroxylation considerably. Liver microsomes prepared from male rats contained higher mephenytoin hydroxylase activity than preparations isolated from females. These results suggest that a cytochrome P-450 which is inducible by pregnenolone 16α-carbonitrile is involved in the 4-hydroxylation of mephenytoin. We purified cytochrome P-450$_{PCN-E}$ from pregnenolone 16α-carbonitrile-treated rats using modifications of previous methods and compared its 4-hydroxylase activity with other purified rat cytochromes P-450. P-450$_{PCN-E}$ had the highest activity among the 10 purified rat cytochromes P-450 tested and antibodies raised to P-450$_{PCN-E}$ completely inhibited mephenytoin 4-hydroxylase in rat liver microsomes, suggesting the involvement of P-450$_{PCN-E}$ in this reaction. The microsomal concentration of P-450$_{PCN-E}$, estimated by immunoelectrophoretic blotting analysis, correlated well with the hydroxylase activity in rat liver microsomes (r = 0.906). Mephenytoin induced P-450$_{PCN-E}$ as well as other phenobarbital-inducible cytochromes P-450.

INTRODUCTION

Interindividual differences in rates of drug metabolism in humans have been recognized for some time (1–3). These differences can be significant in determining the responses of people to certain drugs and their potential side effects (4, 5). Both environmental factors and heredity can be important in determining the amount of each of the enzymes which are involved in drug metabolism.

Mephenytoin is an anti-epileptic drug which shows classic genetic polymorphism in its metabolism in humans (3, 6, 7). The *S*-isomer is hydroxylated at the 4-position, and this reaction is catalyzed by an enzyme (a P-450[3]) which is absent or defective in phenotypic "poor metabolizers." The *R*-isomer is metabolized to nirvanol by an *N*-demethylation pathway which does not appear to be subject to polymorphism (6–9). The mephenytoin 4-hydroxylase polymorphism appears to be distinct from that involved in the oxidation of debrisoquine, sparteine, and other related compounds (10).

In our efforts to understand the mechanism underlying the mephenytoin 4-hydroxylase polymorphism, we sought an experimental animal model. Since a number of P-450s have been characterized in rat liver (11–14), we decided to characterize the particular isozyme involved in mephenytoin 4-hydroxylation in this system, as a first step in examining the suitability of this model. Several lines of evidence presented here indicate that the isozyme involved is the P-450$_{PCN-E}$ previously described by Guzelian and his associates (14), our own group (11, 15), and others (16).[4] Mephenytoin induces this P-450 as well as others.

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[3] The abbreviation used is: P-450, liver microsomal cytochrome P-450.

[4] Several direct comparisons between our own laboratory and that of Dr. Waxman have established that his isozyme "PB-2a" is indistinguishable from our P-450$_{PCN-E}$ (15). In particular, the two enzyme preparations both catalyze testosterone 6β-hydroxylation and warfarin 10-hydroxylation. The testosterone 6β-hydroxylase activity of the "RLM3" preparation of Dr. Schenkman suggests that it might be the same (16). Dr. Guzelian has used the terminology "P-450$_{PCN}$" (14) or "P-450$_p$" (17) since first isolating the protein. We use the term "P-450$_{PCN-E}$" in this report. These comparisons are tentative and await further confirmation but are intended to provide some frame of reference in this field.

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MATERIALS AND METHODS

Mephenytoin, 4-hydroxymephenytoin, and nirvanol were kindly donated by Dr. G. R. Wilkinson of the Department of Pharmacology, Vanderbilt University. Troleandomycin was a gift of Dr. P. S. Guzelian, Medical College of Virginia, Richmond. *S-[methyl-*^{14}C]Mephenytoin was synthesized as described elsewhere (18).

Treatment of animals. Rats of Sprague-Dawley origin (about 100 g) were purchased from Harlan Industries (Indianapolis, IN). Animals were treated with various compounds once each day for 4 days at the following dosages (11): phenobarbital, 100 mg kg^{-1} in 0.15 M NaCl administered intraperitoneally; β-naphthoflavone, 50 mg kg^{-1} in corn oil intraperitoneally; pregnenolone 16α-carbonitrile, 100 mg kg^{-1} in corn oil orally; dexamethasone, 100 mg kg^{-1} in corn oil intraperitoneally; troleandomycin, 200 mg kg^{-1} in corn oil orally (17); and mephenytoin, 100 mg kg^{-1} in corn oil orally. Control (untreated) animals received no chemicals. Animals were killed by decapitation 24 hr after the last administration. Liver microsomes were prepared as described previously (11).

Purification of enzymes. P-450$_{PCN-E}$ was purified to a gel-electrophoretically homogeneous state with a modification of previously reported methods. (As shown later, the catalytic activity seemed to be improved.) Microsomes from pregnenolone 16α-carbonitrile-treated rat livers were solubilized with sodium cholate, and *n*-octylamino-Sepharose 4B chromatography was done as described elsewhere (11), except that the phosphate concentration was reduced from 100 to 10 mM and the first elution of P-450 was carried out with an Emulgen 913 concentration of 0.08%. The peak fraction of P-450 was applied to a 2.6 × 10 cm column of hydroxylapatite which was equilibrated with 10 mM potassium phosphate (pH 7.25) containing 0.1 mM EDTA, 20% (v/v) glycerol, 0.2 mm dithiothreitol, and 0.2% Emulgen 913. The column was washed with 10 and 90 mM phosphate buffers and P-450$_{PCN-E}$ was eluted by increasing the phosphate concentration to 180 mM. Emulgen 913 was removed from the purified preparation by adsorption on a small column of hydroxylapatite, washing with buffer devoid of detergent, and elution with 0.5 M potassium phosphate buffer (pH 7.4) containing 0.1 mM EDTA and 20% (v/v) glycerol. The purified preparation was homogeneous as judged by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, as shown in Fig. 1, and had a specific content of 7–8 nmol of P-450/mg of protein. Cytochrome P-420 accounted for an additional 1–2 nmol (heme)/mg of protein; presumably some apoprotein is present.

Other P-450s, including P-450$_{UT-A}$, P-450$_{PB-B}$, P-450$_{\beta NF-B}$, P-450$_{PB-C}$, P-450$_{PB-D}$, P-450$_{UT-F}$, P-450$_{ISF-G}$, P-450$_{UT-H}$, and P-450$_{UT-I}$, and NADPH-P-450 reductase were purified as described previously (11, 15, 20).

Antisera were raised and treated essentially as described elsewhere (21). In general, "Western" blotting analysis (13) was used as a criterion of specificity (11) and appropriate cross-adsorptions with immobilized microsomes were done to remove unwanted components (15). A recent publication describes the specificity of the key antibody preparations utilized here in the inhibitions of steroid hydroxylations (15).

Analytical methods. The methods for measuring the catalytic activity toward benzphetamine and ethylmorphine are described in the literature (11). Briefly, *S-*[*methyl-*^{14}C]mephenytoin and its 4-hydroxy metabolite were separated by thin layer chromatography on silica using a system of $CHCl_3$/CH_3OH/con NH_4OH (90:10:1) (18). P-450$_{PCN-E}$ in various rat liver microsomal preparations was determined by "Western" blotting analysis as described elsewhere (13). Contents of protein and P-450 were assayed by the method of Lowry *et al.* (22) and Omura and Sato (23), respectively. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis [7.5% (w/v) acrylamide] was carried out as described by Laemmli (24).

RESULTS

Table 1 shows the monooxygenase activities of liver microsomes prepared from rats treated in various ways; the table also includes the protein and P-450 contents in these preparations. All of the inducers increased protein content and P-450 in male rat liver microsomes to various extents. Phenobarbital and β-naphthoflavone caused the most significant increases in P-450 content among the inducers used. Pregnenolone 16α-carbonitrile, dexamethasone, and troleandomycin, which are known inducers of P-450$_{PCN-E}$ in rat liver microsomes (14, 25–28), also increased the P-450 content; the Soret peak of the reduced carbon monoxide complex of the P-450 was changed from 449 nm (from 450 nm) by these treatments. We also found evidence that mephenytoin might induce P-450$_{PCN-E}$ in rat liver microsomes, because the shift of the wavelength maximum of the carbon monoxide complex of P-450 to 449 nm was observed and ethylmorphine

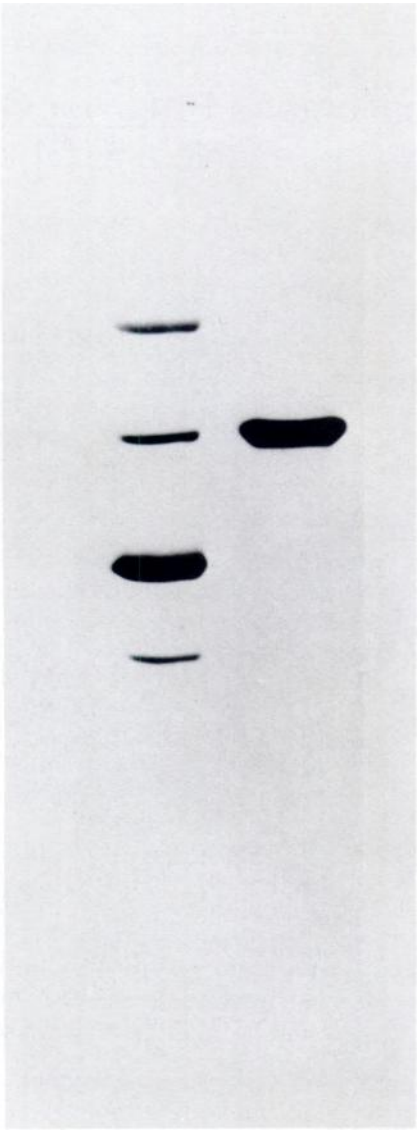

FIG. 1. *Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of P-450*$_{PCN-E}$

Electrophoresis was carried out with 2 μg of P-450$_{PCN-E}$ (right side). The molecular weight standards (left side) were bovine serum albumin (M_r = 68,000), *Escherichia coli* L-glutamate dehydrogenase (M_r = 53,000), equine liver alcohol dehydrogenase (M_r = 43,000), and carbonic anhydrase (M_r = 29,000). The anode was at the bottom of the figure. The gel was stained with a silver method (19).

TABLE 1

Effects of various enzyme inducers on the contents of protein and P-450 and drug oxidation activities in rat liver microsomes

Treatment of rats with various inducers and determination of microsomal components and enzyme activities were performed as described in Materials and Methods. Results are expressed as means of experiments done with two animals ± standard deviation (*i.e.*, range).

Treatment	Protein	P-450	Demethylase activity		Mephenytoin 4-hydroxylase,activity	
			Ethylmorphine	Benzphetamine		
	mg/g liver	*nmol/mg*	*nmol HCHO/min/mg protein*		*pmol/min/mg protein*	*pmol/min/nmol P-450*
Female						
None	20.1 ± 0.6	0.32 ± 0.06	1.08 ± 0.09	ND[a]	15 ± 1	47 ± 4
Male						
None	20.1 ± 0.9	0.61 ± 0.02	2.73 ± 0.17	1.93 ± 0.27	37 ± 5	62 ± 10
Phenobarbital	25.6 ± 1.8	1.53 ± 0.10	4.95 ± 0.30	4.18 ± 0.11	136 ± 1	89 ± 6
β-Naphthoflavone	24.1 ± 1.7	1.64 ± 0.47	2.13 ± 0.62	ND	60 ± 32	35 ± 10
Pregnenolone 16α-carbonitrile	32.6 ± 5.3	0.92 ± 0.09	3.90 ± 1.16	ND	117 ± 21	129 ± 36
Dexamethasone	30.0 ± 9.5	0.98 ± 0.04	6.28 ± 1.62	ND	198 ± 14	202 ± 23
Troleandomycin	26.6 ± 1.8	0.69 ± 0.20	8.14 ± 0.40	ND	138 ± 25	203 ± 20
Mephenytoin	34.8 ± 2.5	0.98 ± 0.11	7.68 ± 0.74	5.59 ± 0.35	156 ± 21	160 ± 38

[a] Not determined.

N-demethylase activity (which is one of the measures of P-450$_{PCN-E}$ in liver microsomes) was significantly enhanced by mephenytoin treatment (14).

Ethylmorphine *N*-demethylase activity was also increased with phenobarbital, pregnenolone 16α-carbonitrile, dexamethasone, and troleandomycin, but not with β-naphthoflavone. Liver microsomes prepared from male rats had higher ethylmorphine demethylase activity than those prepared from females. The pattern of inducing effects of demethylation activity by various chemicals is very similar to the mephenytoin 4-hydroxylase activity. Inducers of P-450$_{PCN-E}$ can significantly enhance mephenytoin 4-hydroxylation and microsomes prepared from male rats gave higher turnover numbers than those prepared from females. Dexamethasone was the most effective inducer, followed by mephenytoin, troleandomycin, phenobarbital, and pregnenolone 16α-carbonitrile. On the basis of P-450 content, pregnenolone 16α-carbonitrile treatment resulted in a much higher induction of hydroxylase activity than did phenobarbital treatment. Benzphetamine *N*-demethylase activity was induced by treatment of male rats with both phenobarbital and mephenytoin. These results first suggested that P-450$_{PCN-E}$ might participate in the metabolism of mephenytoin in rat liver microsomes.

Table 2 shows the mephenytoin 4-hydroxylase activities of reconstituted monooxygenase systems containing each of 10 forms of rat liver P-450. We detected the enzyme activity only with P-450$_{PCN-E}$ and P-450$_{UT-A}$; the former P-450 can catalyze about 3-fold higher activity than the latter. The increase in enzyme activity by purified P-450$_{PCN-E}$ as compared with liver microsomes was about 3-fold. P-450$_{PCN-E}$ has been shown to lose ethylmorphine demethylase activity after purification from pregnenolone 16α-carbonitrile-treated rat liver microsomes (14), as well as warfarin 10-hydroxylase (11) and testosterone 6β-hydroxylase activities (15).

To further study the possible participation of P-450$_{PCN-E}$ in the metabolism of mephenytoin in rat liver microsomes, rabbit antisera raised to purified P-450$_{PCN-E}$ were used to determine the contents of P-450$_{PCN-E}$ in microsomes, and the correlation between P-450$_{PCN-E}$ content and mephenytoin 4-hydroxylase activity was examined. As shown in Fig. 2, the contents of P-450$_{PCN-E}$ and mephenytoin 4-hydroxylase activities were correlated very well ($r = 0.906$).

TABLE 2

Mephenytoin 4-hydroxylase activity of monooxygenase systems containing each of 10 forms of rat P-450

Reaction mixtures (0.1 ml) contained 0.02–0.05 nmol of P-450, 0.25 nmol of NADPH-P-450 reductase, 2 μg of L-α-dilauroyl-3-phosphatidylcholine, and an NADPH-generating system (11). Reactions were done at 37° for 30 min, and the product formation was determined by the method described previously. Data are means of two determinations ± standard deviation (*i.e.*, range).

P-450 isozyme	Mephenytoin 4-hydroxylation
	nmol/min/nmol P-450
UT-A	0.13 ± 0.02
BNF-B	<0.005
PB-B	<0.005
PB-C	<0.005
PB-D	<0.005
PCN-E	0.38 ± 0.08
UT-F	<0.005
ISF-G	<0.005
UT-H	<0.005
UT-I	<0.005

Fig. 3 shows the effects of a rabbit anti-rat P-450$_{PCN-E}$ immunoglobulin G fraction on the mephenytoin 4-hydroxylase activity in rat liver microsomes. Anti-P-450$_{PCN-E}$ completely inhibited the hydroxylase activity in liver microsomes prepared from untreated or pregnenolone 16α-carbonitrile-treated male rats. The effect of anti-P-450$_{UT-A}$ was not very significant. Antisera raised to the other rat P-450s did not inhibit mephenytoin 4-hydroxylase activity.[5]

[5] Crude anti-P-450$_{UT-I}$ inhibited activity but the inhibitory effect could be removed by adsorption of the antisera with immobilized microsomes prepared from phenobarbital-treated male rats.

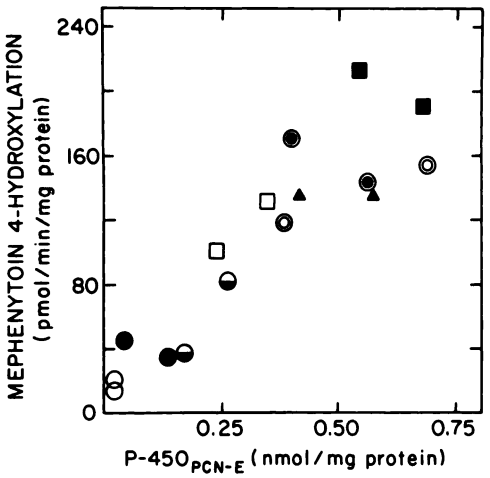

FIG. 2. *Correlation of the contents of P-450$_{PCN-E}$ and the mephenytoin 4-hydroxylase activity in various rat liver microsomes*

Liver microsomes from untreated female (○), untreated male (●), phenobarbital-treated male (▲), β-naphthoflavone-treated male (◒), pregnenolone 16α-carbonitrile-treated male (□), dexamethasone-treated male (■), troleandomycin-treated male (⊙), and mephenytoin-treated male (◉) rats were used. Each point represents determinations made with a single animal. The correlation coefficient (r) is 0.906, the slope is 0.25 nmol of 4-hydroxymephenytoin produced/min/nmol of P-450, and the y intercept is 23 pmol of 4-hydroxymephenytoin produced/min/mg of microsomal protein.

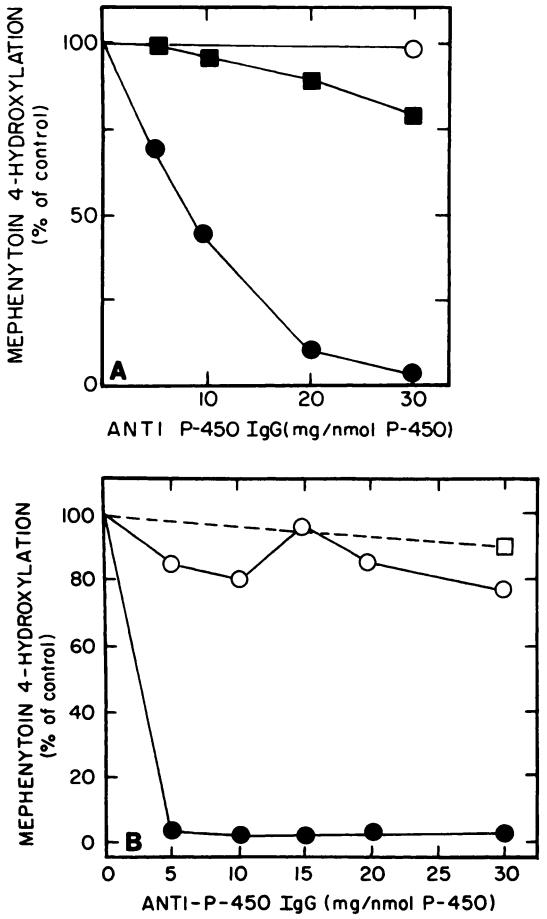

FIG. 3. *Inhibition of mephenytoin 4-hydroxylase activity by anti-P-450$_{PCN-E}$*

A, the indicated amounts of the IgG fractions of rabbit anti-P-450$_{PCN-E}$ (●) or anti-P-450$_{UT-A}$ (■) were added to the reaction mixture containing liver microsomes from pregnenolone 16α-carbonitrile-treated male rats. The effect of a preimmune IgG fraction (○) was also measured. The uninhibited hydroxylase activity fraction was 0.35 nmol/min/nmol of P-450 in this instance (pregnenolone 16α-carbonitrile-treated rats). B, the indicated amounts of the IgG fractions of rabbit anti-P-450$_{PCN-E}$ (●) or anti-P-450$_{UT-A}$ (○) were added to the reaction mixture containing liver microsomes prepared from untreated male rats. The effect of a preimmune IgG fraction was also measured (□).

DISCUSSION

Several lines of evidence support the view that P-450$_{PCN-E}$ is the major P-450 isozyme involved in mephenytoin 4-hydroxylation in male rats and in female rats which have been treated with certain barbiturates, steroids, or antibiotics or with mephenytoin itself. First, out of 10 purified P-450 isozymes which were tested in reconstituted systems, P-450$_{PCN-E}$ was most active in catalyzing mephenytoin 4-hydroxylation (Table 1). In these studies the *apparent* turnover number was approximately as high in the reconstituted system as in the liver microsomes when the immunochemically determined amount of P-450$_{PCN-E}$ was used as a basis for comparison (Fig. 2). This result is unusual in consideration of the low activities of other purified preparations of this enzyme in other assays (11, 14, 15). Attempts to enhance the catalytic activity further with cytochrome b_5 were inconclusive; no efforts were made to insert the enzyme into phospholipid vesicles. The second line of evidence implicating P-450$_{PCN-E}$ was the immunochemical inhibition work (Fig. 3). The inhibition was clearly greatest with anti-P-450$_{PCN-E}$. Anti-P-450$_{UT-A}$ showed only slight inhibition; these data, coupled with the reconstitution results (Table 2), suggest a small role for P-450$_{UT-A}$.

The patterns of mephenytoin 4-hydroxylase activity in rat liver microsomes are also consonant with the assignment of this activity to P-450$_{PCN-E}$. P-450$_{PCN-E}$ is male-specific in adult rats (young females have P-450$_{PCN-E}$ and the isozyme nearly disappears during puberty (15)). Table 1 shows the sex difference in rats. In other experiments with older females, we found even less mephenytoin hydroxylase activity. Mephenytoin 4-hydroxylase was not elevated by treatment with β-naphthoflavone but was increased by compounds known to induce P-450$_{PCN-E}$. The level of mephenytoin 4-hydroxylase activity was correlated somewhat with ethylmorphine *N*-demethylase, a fair but not perfect marker for P-450$_{PCN-E}$ (11, 14). More convincing evidence comes from the direct correlation of mephenytoin 4-hydroxylase activity with immunochemically estimated levels of P-450$_{PCN-E}$ (Fig. 2).

Mephenytoin induced its own 4-hydroxylation by increasing levels of P-450$_{PCN-E}$ (Table 1). In a preliminary study, we found that mephenytoin also induced P-450$_{PB-B}$ and P-450$_{PB-C}$ to the same extents as did phenobarbital by Western blotting analysis. Table 1 shows that benzphetamine demethylase activity was increased by mephenytoin treatment. Guzelian *et al.* (28) previously reported that mephenytoin induced *de novo* synthesis of P-450$_{PB-B}$ (or a closely related isozyme) in rat hepatocyte cultures but did not induce P-450$_{PCN-E}$.

Presumably, this difference from the *in vivo* situation represents the loss of regulatory elements, as phenobarbital did not induce $P\text{-}450_{PCN\text{-}E}$ in such cultures either.

In rats, the mephenytoin 4-hydroxylase activity appears to be dissociated from debrisoquine 4-hydroxylation, which is a property of $P\text{-}450_{UT\text{-}H}$ (20), consonant with *in vitro* human results. The further adequacy of the rat as a model for mephenytoin 4-hydroxylase in humans is unknown. Our preliminary studies with blotting analysis indicate that rabbit anti-rat $P\text{-}450_{PCN\text{-}E}$ reacts with human cytochromes $P\text{-}450_2$, $P\text{-}450_3$, $P\text{-}450_4$, $P\text{-}450_5$, and $P\text{-}450_7$, a group of very similar proteins purified previously (29), and antibodies raised to these human proteins recognize rat $P\text{-}450_{PCN\text{-}E}$ (as opposed to $P\text{-}450_{PB\text{-}D}$, as incorrectly suggested in that reference). However, neither anti-$P\text{-}450_{PCN\text{-}E}$ nor antibodies raised to the human cytochromes P-450 mentioned above inhibited mephenytoin 4-hydroxylation in human liver microsomes. Significant sex differences in mephenytoin 4-hydroxylation in humans have not been reported, nor has the ability of barbiturates, hydantoins, or glucocorticoid steroids to increase metabolism. Rats have a tendency to show unusual sex differences in the metabolism of drugs, and this model may not be appropriate. The usefulness of the rat enzyme model may require more knowledge concerning biochemical aspects of the human enzyme.

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REFERENCES

1. Mahgoub, A., J. R. Idle, L. G. Dring, R. Lancaster, and R. L. Smith. Polymorphic hydroxylation of debrisoquine in man. *Lancet* **2**:584–586 (1977).
2. Scott, J., and P. L. Poffenbarger. Pharmacogenetics of tolbutamide metabolism in humans. *Diabetes* **28**:41–51 (1979).
3. Küpfer, A., P. Desmond, R. Patwardhan, S. Schenker, and R. Branch. Mephenytoin hydroxylation deficiency: kinetics after repeated doses. *Clin. Pharmacol. Ther.* **35**:33–39 (1984).
4. Idle, J. R., and R. L. Smith. Polymorphisms of oxidation at carbon centers of drugs and their clinical significance. *Drug Metab. Rev.* **9**:301–317 (1979).
5. Eichelbaum, M. Defective oxidation of drugs: pharmacokinetics and therapeutic implications. *Clin. Pharmacokinet.* **7**:1–22 (1982).
6. Küpfer, A., and R. Preisig. Pharmacogenetics of mephenytoin: a new drug hydroxylation polymorphism in man. *Eur. J. Clin. Pharmacol.* **26**:753–759 (1984).
7. Troupin, A. S., L. Moretti-Ojemann, and C. B. Dodrill. Stereoselective metabolism of mephenytoin in man. *Epilepsia* **17**:403–414 (1976).
8. Küpfer, A., R. K. Roberts, S. Schenker, and R. A. Branch. Stereoselective metabolism of mephenytoin in man. *J. Pharmacol. Exp. Ther.* **218**:193–199 (1981).
9. Küpfer, A., G. M. Brills, J. T. Watson, and T. M. Harris. A major pathway of mephenytoin metabolism in man: aromatic hydroxylation to *p*-hydroxymephenytoin. *Drug Metab. Dispos.* **8**:1–4 (1980).
10. Jurima, M., T. Inaba, and W. Kalow. Sparteine oxidation by the human liver: absence of inhibition by mephenytoin. *Clin. Pharmacol. Ther.* **35**:426–428 (1984).
11. Guengerich, F. P., G. A. Dannan, S. T. Wright, M. V. Martin, and L. S. Kaminsky. Purification and characterization of rat liver microsomal cytochromes P-450: electrophoretic, spectral, catalytic, and immunochemical properties and inducibility of eight isozymes isolated from rats treated with phenobarbital or β-naphthoflavone. *Biochemistry* **21**:6019–6030 (1982).
12. Ryan, D. E., S. Iida, A. W. Wood, P. E. Thomas, C. S. Lieber, and W. Levin. Characterization of three highly purified cytochromes P-450 from hepatic microsomes of adult male rats. *J. Biol. Chem.* **259**:1239–1250 (1984).
13. Guengerich, F. P., P. Wang, and N. K. Davidson. Estimation of isozymes of microsomal cytochrome P-450 in rats, rabbits, and humans using immunochemical staining coupled with sodium dodecyl sulfate-polyacrylamide gel electrophoresis. *Biochemistry* **21**:1698–1706 (1982).
14. Elshourbagy, N., and P. S. Guzelian. Separation, purification, and characterization of a novel form of hepatic cytochrome P-450 from rats treated with pregnenolone-16α-carbonitrile. *J. Biol. Chem.* **255**:1279–1285 (1980).
15. Waxman, D. J., G. A. Dannan, and F. P. Guengerich. Age-dependent expression, hormonal imprinting, and xenobiotic inducibility of sex-specific cytochrome P-450 isozymes. *Biochemistry* in press (1985).
16. Cheng, K-C., and J. B. Schenkman. Purification and characterization of two constitutive forms of rat liver microsomal cytochrome P-450. *J. Biol. Chem.* **257**:2378–2385 (1982).
17. Wrighton, S. A., P. Maurel, E. G. Schuetz, P. B. Watkins, B. Young, and P. S. Guzelian. Identification of the cytochrome P-450 induced by macrolide antibiotics in rat liver as the glucocorticoid responsive cytochrome P-450p. *Biochemistry* **24**:2171–2178 (1985).
18. Shimada, T., J. P. Shea, and F. P. Guengerich. A convenient assay for mephenytoin 4-hydroxylase activity of human liver microsomal cytochrome P-450. *Anal. Biochem.* **147**:174–179 (1985).
19. Wray, W., T. Boulikas, V. P. Wray, and R. Hancock. Silver staining of proteins in polyacrylamide gels. *Anal. Biochem.* **118**:197–203 (1981).
20. Larrey, D., L. M. Distlerath, G. A. Dannan, G. R. Wilkinson, and F. P. Guengerich. Purification and characterization of the rat liver microsomal cytochrome P-450 involved in the 4-hydroxylation of debrisoquine, a prototype for genetic variation in oxidative drug metabolism. *Biochemistry* **23**:2787–2795 (1984).
21. Kaminsky, L. S., M. J. Fasco, and F. P. Guengerich. Production and application of antibodies to rat liver cytochrome P-450. *Methods Enzymol.* **74**:262–272 (1981).
22. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**:265–275 (1951).
23. Omura, T., and R. Sato. The carbon monoxide-binding pigment of liver microsomes. I. Evidence for its hemoprotein nature. *J. Biol. Chem.* **239**:2370–2385 (1964).
24. Laemmli, U. K. Cleavage of structural proteins during the assembly of the head of bacteriophage T_4. *Nature (Lond.)* **227**:680–686 (1970).
25. Lu, A. Y. H., A. Smogyi, S. West, R. Kuntzman, and A. H. Conney. Pregnenolone-16α-carbonitrile: a new type of inducer of drug metabolizing enzymes. *Arch. Biochem. Biophys.* **152**:457–462 (1972).
26. Schuetz, E. G., S. A. Wrighton, J. C. Barwick, and P. S. Guzelian. Induction of cytochrome P-450 by glucocorticoids in rat liver. I. Evidence that glucocorticoids and pregnenolone 16α-carbonitrile regulate *de novo* synthesis of a common form of cytochrome P-450 in cultures of adult rat hepatocytes and in the liver *in vivo*. *J. Biol. Chem.* **259**:1999–2006 (1984).
27. Heuman, D. M., E. J. Gallagher, J. C. Barwick, N. Elshourbagy, and P. S. Guzelian. Immunochemical evidence for induction of a common form of hepatic cytochrome P-450 in rats treated with pregnenolone-16α-carbonitrile or other steroidal or non-steroidal agents. *Mol. Pharmacol.* **21**:753–760 (1982).
28. Guzelian, P. S., S. Newman, and E. Gallagher. Stimulated *de novo* synthesis of cytochromes P-450 by drugs and steroids in primary monolayer cultures of adult rat hepatocytes, in *Cytochrome P-450: Biochemistry, Biophysics, and Environmental Implications* (E. Hietanen, M. Laitenen, and O. Hanninen, eds.). Elsevier, New York, 153–156 (1982).
29. Wang, P. P., P. Beaune, L. S. Kaminsky, G. A. Dannan, F. F. Kadlubar, D. Larrey, and F. P. Guengerich. Purification and characterization of six cytochrome P-450 isozymes from human liver microsomes. *Biochemistry* **22**:5375–5383 (1983).

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