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Characterization of Rat and Human Liver Microsomal Cytochrome P-450 Forms Involved in Nifedipine Oxidation, a Prototype for Genetic Polymorphism in Oxidative Drug Metabolism*

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The metabolism of the dihydropyridine calcium antagonist and vasodilator nifedipine has been reported to exhibit polymorphism among individual humans (Kleinbloesem, C. H., van Brummelen, P., Faber, H., Danhof, M., Vermeulen, N. P. E., and Breimer, D. D. (1984) *Biochem. Pharmacol.* 33, 3721–3724). Nifedipine oxidation has been shown to be catalyzed by cytochrome P-450 (P-450) enzymes. Reconstitution, immunoinhibition, and induction studies with rat liver indicated that the forms designated P-450$_{UT-A}$ and P-450$_{PCN-E}$ are the major contributors to microsomal nifedipine oxidation. The P-450 which oxidizes nifedipine (P-450$_{NF}$) was purified to electrophoretic homogeneity from several human liver samples. Antibodies raised to P-450$_{NF}$ were highly specific as judged by immunoblotting analysis and inhibited >90% of the nifedipine oxidase activity in human liver microsomes. A monoclonal antibody raised to the human P-450 preparation reacted with both human P-450$_{NF}$ and rat P-450$_{PCN-E}$. Immunoblotting analysis of 39 human liver microsomal samples using anti-P-450$_{NF}$ antibodies revealed the same 52,000-dalton polypeptide, corresponding to P-450$_{NF}$, with only one of the microsomal samples showing an additional immunoreactive protein. The level of nifedipine oxidase activity was highly correlated with the amount of P-450$_{NF}$ thus detected using either polyclonal (r = 0.78) or monoclonal (r = 0.65) antibodies, suggesting that the amount of the P-450$_{NF}$ polypeptide may be a major factor in influencing the level of catalytic activity in humans as well as rats. Cytochrome b_5 enhanced the catalytic activity of reconstituted P-450$_{NF}$, and anti-cytochrome b_5 inhibited nifedipine oxidase activity in human liver microsomes. P-450$_{NF}$ also appears to be a major contributor to human liver microsomal aldrin epoxidation, *d*-benzphetamine *N*-demethylation, 17β-estradiol 2- and 4-hydroxylation, and testosterone 6β-hydroxylation, the major pathway for oxidation of this androgen in human liver microsomes.

Interindividual variations in the oxidative metabolism of drugs have been recognized and are probably due at least in part to polymorphisms in the cytochrome P-450[1] enzymes (Küpfer and Preisig, 1984; Guengerich *et al.*, 1986). These polymorphisms usually contain a strong genetic component and, in addition to predisposing individuals to potential drug toxicities, may also contribute to the influence of host factors in carcinogenesis, as the P-450s are involved in the biotransformation of a variety of environmental pollutants, pesticides, and cancer-causing agents as well as drugs (Wislocki *et al.*, 1980). Such polymorphisms have been characterized in animal models (Simmons and Kasper, 1983; Larrey *et al.*, 1984; Johnson and Schwab, 1984) but only recently have the molecular details been considered in humans. We have recently purified human P-450s involved in three of these polymorphisms, namely debrisoquine 4-hydroxylation, phenacetin *O*-deethylation, and mephenytoin 4-hydroxylation (Distlerath *et al.*, 1985; Guengerich *et al.*, 1986; Shimada *et al.*, 1986). In each of these polymorphisms about 5% of the individuals in Caucasian populations are phenotyically deficient in activity, although the fraction varies with race (Kalow, 1984).

Recently Kleinbloesem *et al.* (1984) identified an apparently bimodal variation in the metabolism of the vasodilator and calcium agonist nifedipine. In that study 17% of the individuals examined were phenotypically deficient in the first step

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[1] The abbreviations used are: P-450, liver microsomal cytochrome P-450; $NaDodSO_4$, sodium dodecyl sulfate; IgG, immunoglobulin G; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; HPLC, high performance liquid chromatography. The terminology "HL" is used here to denote human liver microsomal preparations, which are identified by code number. The nomenclature used for the rat P-450 isozyme and comparison to preparations of others has been made elsewhere (Guengerich *et al.*, 1982a; Waxman *et al.*, 1985). Of particular note here, P-450$_{UT-A}$ (Guengerich *et al.*, 1982a) appears to correspond to preparations designated "male-specific P-450" (Kamataki *et al.*, 1983), "RLM 5" (Cheng and Schenkman, 1982), "P-450 2c" (Waxman *et al.*, 1983; Waxman, 1984), "h" (Haniu *et al.*, 1984), and "P-450 A" (LeProvost *et al.*, 1983). P-450$_{PCN-E}$ (Guengerich *et al.*, 1982a, 1982b) appears to correspond to other preparations designated "P-450$_{PCN}$" (Elshourbagy and Guzelian, 1980) and "PB-2a" (Waxman, 1984). In some cases where experiments in this report were done with preparations of these P-450s made in Dr. Waxman's laboratory, a combined designation is used (*e.g.* P-450$_{PCN-E(PB-2a)}$). The human P-450s are designated P-450$_{NF}$ (this report), P-450$_{DB}$ (Distlerath *et al.*, 1985), P-450$_{PA}$ (Distlerath *et al.*, 1985), and P-450$_{MP}$ (Guengerich *et al.*, 1986) to signify their respective involvements in the genetic polymorphisms of nifedipine oxidation (NF), debrisoquine 4-hydroxylation (DB), phenacetin *O*-deethylation (PA), and *S*-mephenytoin-4-hydroxylation (MP) activities.

TABLE IV
Nifedipine oxidase activities of rat liver microsomes

	Sex of rats	Treatment of rats	Nifedipine oxidase activity	
			nmol product formed/min/mg microsomal protein	nmol product formed/min/nmol P-450
Experiment I[a]	Female		0.25	0.25
	Female	Phenobarbital	1.63	0.46
	Male		5.34	5.51
	Male	Phenobarbital	13.2	3.91
	Male	β-Naphthoflavone	1.80	1.60
	Male	Isosafrole	7.96	3.79
	Male	Aroclor 1254	8.03	1.76
Experiment II[b]	Male		1.65 (± 0.21)	2.71 (± 0.35)
	Male	Pregnenolone 16α-carbonitrile	2.34 (± 0.57)	2.52 (± 0.61)
	Male	Dexamethasone	4.16 (± 0.98)	4.24 (± 1.05)
	Male	Triacetyloleandomycin	2.28 (± 0.32)	3.25 (± 0.45)

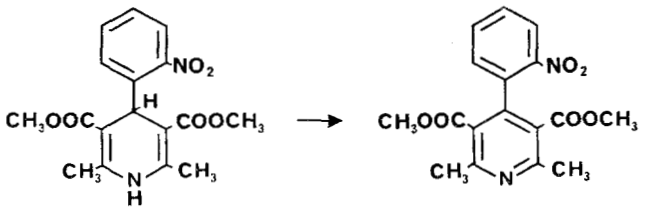
[a] Results are expressed as means of duplicate assays.
[b] Results are expressed as means (± S.D.) of triplicate assays. In these studies the rats weighed only 75–100 g, and the lower catalytic activity (compared to Experiment I) is attributed to lack of development of P-450$_{UT-A}$ (Waxman *et al.*, 1985).

of metabolism, the formal 2-electron oxidation of the nifedipine dihydropyridine ring (Scheme 1). Such an oxidation could in principle be catalyzed by a number of different oxidoreductases, but some precedent exists for the involvement of P-450s (Augusto *et al.*, 1982). The primary metabolite is further transformed by saponification of the ester and hydroxylation of the ring methyls (Raemsch and Sommer, 1983).

In this report we identify the P-450 forms in rat liver which are responsible for nifedipine oxidation. The human liver P-450 involved in this activity has also been identified, purified to apparent homogeneity, and partially characterized with regard to its role in the interindividual variation. Preliminary reports of these findings have been presented in abstract form (Guengerich *et al.*, 1985).

EXPERIMENTAL PROCEDURES AND RESULTS[2]

Nifedipine Oxidase Activity in Rat Liver Microsomes—In order to define which rat P-450s contribute to microsomal nifedipine oxidation, the effects of sex and monooxygenase induction agents on rat liver microsomal nifedipine metabolism were examined (Table IV). Microsomes prepared from untreated males oxidized nifedipine 20-fold faster than did those prepared from untreated females. On a per mg protein basis, the activity could be increased by treatment with phenobarbital in either sex or with isosafrole or the polychlorinated biphenyl mixture Aroclor 1254. Administration of β-naphthoflavone (which both induces P-450$_{\beta NF-B}$ and P-450$_{ISF-G}$ and suppresses the male-specific P-450$_{UT-A}$ (Guengerich *et al.*, 1982a)) decreased nifedipine oxidase activity. Pregnenolone 16α-carbonitrile, dexamethasone, and triacetyloleandomycin, which all induce P-450$_{PCN-E}$, all elevated nifedipine oxidase activity (Table IV, Experiment II). Of the phenobarbital-inducible P-450s, only P-450$_{PCN-E}$ is male specific in untreated rats (Waxman *et al.*, 1985).



SCHEME 1. **Oxidation of nifedipine to its pyridine metabolite.**

Catalytic Activities of Purified Rat P-450 Isozymes—Ten different purified rat P-450s were assayed for nifedipine oxidase activity after reconstitution with rat NADPH-P-450 reductase and phospholipid (Table V). P-450$_{UT-A}$ clearly exhibited the highest catalytic activity but P-450$_{PCN-E}$ and P-450$_{PB-B}$ also had some activity. P-450$_{PCN-E}$, apparently alone among the rat P-450s studied to date, is known to lose catalytic activity during purification (Elshourbagy and Guzelian, 1980; Guengerich *et al.*, 1982a; Shimada and Guengerich, 1985; Waxman *et al.*, 1985).

Immunochemical Inhibition of Catalytic Activity in Liver Microsomes—The studies with microsomes and reconstituted P-450 systems described above suggested that P-450$_{UT-A}$, P-450$_{PCN-E}$, and P-450$_{PB-B}$ might be the major forms of P-450 contributing to nifedipine oxidation in rat liver. In order to examine the roles of these enzymes more closely, inhibitory antibodies specific for each of these P-450s were incubated with microsomes prepared from untreated male rats (Fig. 10). Both anti-P-450$_{UT-A}$ and anti-P-450$_{PCN-E}$ showed extensive inhibition, but anti-P-450$_{PB-B}$ did not. Moreover, anti-P-450$_{PB-B}$ did not inhibit nifedipine oxidase activity in liver microsomes prepared from phenobarbital-treated adult female rats (which contain P-450$_{PCN-E}$ and P-450$_{PB-B}$ but not P-450$_{UT-A}$ (Waxman *et al.*, 1985)) (data not shown).

Purification, Properties, and Reconstitution of Human Liver P-450$_{NF}$—P-450$_{NF}$ was isolated from several human liver samples as described in the Miniprint using a high performance liquid chromatography assay to monitor nifedipine oxidase activity. Also detailed in the Miniprint are the electrophoretic, spectral, and immunochemical properties of P-450$_{NF}$ and studies on its catalytic specificity.

Anti-P-450$_{NF}$ was found to almost completely inhibit nifedipine oxidation in human liver microsomes (Fig. 11). Anti-

[2] Portions of this paper (including "Experimental Procedures," part of "Results," Tables I–III, Figs. 1–9, and Footnotes 3–6) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are available from the Journal of Biological Chemistry, 9650 Rockville Pike, Bethesda, MD 20814. Request Document No. 85M-2478, cite the authors, and include a check or money order for $11.60 per set of photocopies. Full size photocopies are also included in the microfilm edition of the Journal that is available from Waverly Press.

TABLE V

Nifedipine oxidase activities of purified rat P-450 preparations

P-450 isozyme	Nifedipine oxidase activity
	nmol product formed/min/nmol P-450
$P\text{-}450_{UT\text{-}A}$	3.5
$P\text{-}450_{PB\text{-}B}$	0.39
$P\text{-}450_{\beta NF\text{-}B}$	0.15
$P\text{-}450_{PB\text{-}C}$	<0.03
$P\text{-}450_{PB\text{-}D}$	0.15
$P\text{-}450_{PCN\text{-}E}$	0.66
$P\text{-}450_{UT\text{-}F}$	0.06
$P\text{-}450_{ISF\text{-}G}$	<0.03
$P\text{-}450_{UT\text{-}H}$	0.13
$P\text{-}450_{UT\text{-}I}$	<0.03

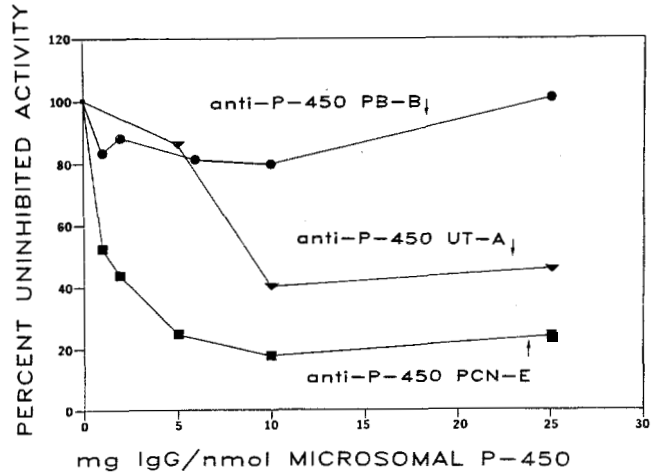


FIG. 10. **Immunoinhibition of rat liver microsomal nifedipine oxidase activity.** Liver microsomes prepared from untreated male rats were assayed for nifedipine oxidase activity after preincubation with IgG fractions prepared from rabbit anti-$P\text{-}450_{PB\text{-}B}$ (●), anti-$P\text{-}450_{UT\text{-}A}$ (▼), or anti-$P\text{-}450_{PCN\text{-}E}$ (■). Preimmune IgG was added to each incubate to give a total IgG level of 25 mg/nmol P-450 in each case. Incubations contained 52 pmol of microsomal P-450, and the incubation time was 15 min. The uninhibited activity was 4.4 nmol of product formed/min/nmol of P-450. The indicated points represent means of duplicate experiments.

rat $P\text{-}450_{UT\text{-}A}$ did not inhibit, but anti-rat $P\text{-}450_{PCN\text{-}E}$ was somewhat inhibitory in human as well as rat liver microsomes (Fig. 11).

Role of Cytochrome b_5 in Human Microsomal Nifedipine Oxidase Activity—Preliminary studies indicated that the inclusion of either purified rat or human liver cytochrome b_5 in the reconstituted $P\text{-}450_{NF}$ resulted in a variable enhancement of nifedipine oxidase activity. The influence of cytochrome b_5 on three distinct purified $P\text{-}450_{NF}$ preparations was, therefore, examined in greater detail (Fig. 12). While a positive effect was observed with each $P\text{-}450_{NF}$ preparation, the extent of stimulation and the amount of cytochrome b_5 required for the maximal effect were rather variable.

In order to gain more insight into the role of cytochrome b_5, antibodies were raised against purified cytochrome b_5 isolated from liver sample HL91. The antibodies recognized a single polypeptide in each of three human microsomal preparations as demonstrated by immunoblotting analysis and also inhibited cytochrome b_5-dependent (Omura and Takesue, 1970) NADH-cytochrome-*c* reductase activity in human liver microsomes. Although the inhibition of microsomal nifedipine oxidase activity required large amounts of anti-cytochrome b_5 IgG, parallel inhibition of cytochrome b_5-dependent NADH-cytochrome *c* reduction was evident (Fig. 13).

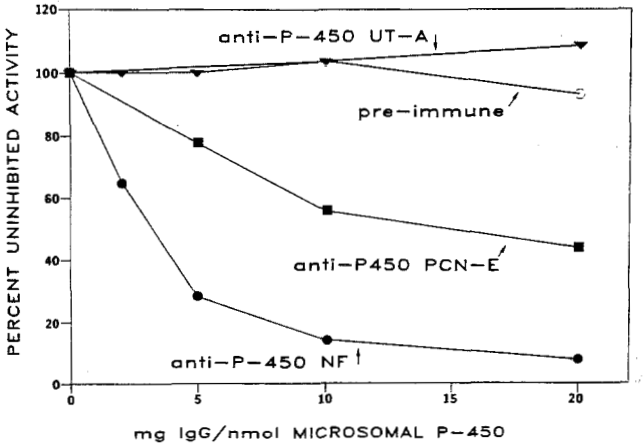


FIG. 11. **Immunoinhibition of human liver microsomal nifedipine oxidase activity.** Human liver microsomes (sample HL92) were assayed for nifedipine oxidase activity after preincubation with IgG fractions prepared from rabbit preimmune sera (○), anti-rat $P\text{-}450_{UT\text{-}A}$ (▼), anti-rat $P\text{-}450_{PCN\text{-}E}$ (■), or $P\text{-}450_{NF}$ (●). The IgG preparations were the same used in Fig. 10. Incubations contained 50 pmol of microsomal P-450, and the incubation time was 10 min. The uninhibited activity was 6.7 nmol of product formed/min/nmol of P-450. The indicated points represent means of duplicate experiments. Similar patterns of inhibition with anti-$P\text{-}450_{NF}$ have been observed in several other human liver microsomal preparations (data not shown).

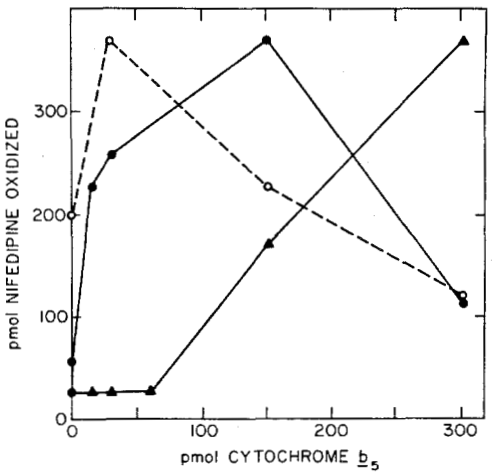


FIG. 12. **Enhancement of nifedipine oxidase activity of purified human $P\text{-}450_{NF}$ by human liver cytochrome b_5.** Reconstituted systems containing 50 pmol of $P\text{-}450_{NF}$ isolated from liver sample HL37 (○), HL92 (●), or HL93 (▲), 250 pmol of rabbit NADPH-P-450 reductase, and 15 nmol of L-α-1,2-dilauroyl-*sn*-glycero-3-phosphocholine were fortified with the indicated amounts of purified human liver cytochrome b_5 (added as a 1 μM solution 30 min after mixing the previous components at 23 °C). After 10 min, other reaction components were added (total volume, 0.5 ml), and the reactions were incubated for 5 min.

Correlation of Nifedipine Oxidase Activity with Immunochemically Determined Concentrations of $P\text{-}450_{NF}$—The levels of nifedipine oxidase activity in individual human liver microsomal preparations were compared to the immunochemically estimated levels of $P\text{-}450_{NF}$ (Fig. 14). When the comparison was made using rabbit anti-$P\text{-}450_{NF}$, the correlation was highly significant ($r = 0.78$, $n = 32$, $p < 0.005$). When a similar comparison was made using a monoclonal antibody raised to human liver $P\text{-}450_5$ (Wang *et al.*, 1983; Beaune *et al.*, 1985), the correlation was only slightly less significant ($r = 0.65$, $n = 39$, $p < 0.005$).

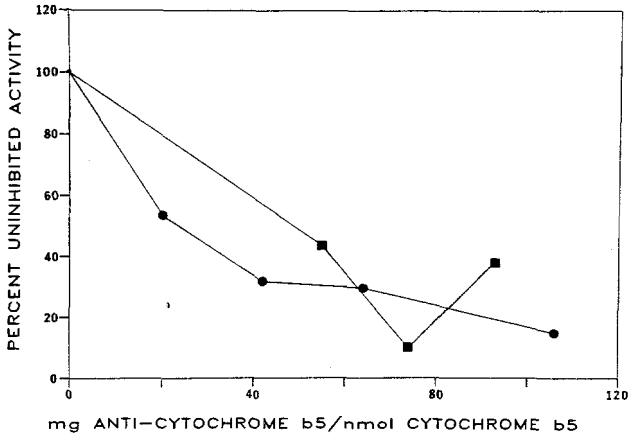


FIG. 13. **Immunoinhibition of human liver microsomal nifedipine oxidase activity by anti-human liver cytochrome b_5.** Human liver microsomes (sample HL92) were assayed for NADH-cytochrome *c* reductase activity (●) and nifedipine oxidase activity (▼) after 60 min of preincubation at 23 °C with the indicated amount of the IgG fraction of rabbit-anti-human liver cytochrome b_5. The mixtures were adjusted with the IgG fraction from preimmune antiserum such that all contained 110 mg of *total* IgG/nmol of microsomal b_5. The indicated points represent means of duplicate experiments.

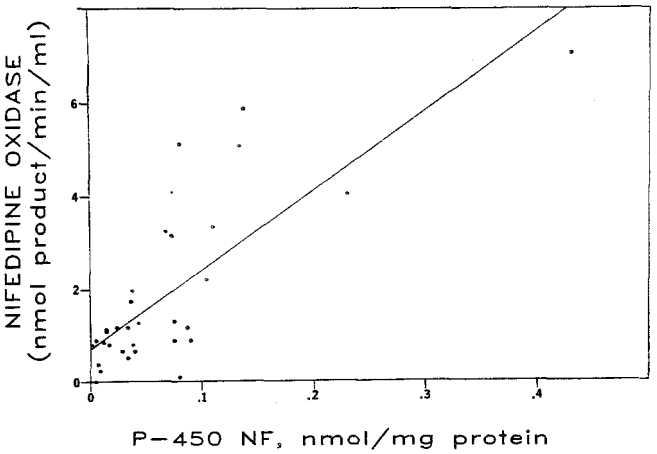


FIG. 14. **Correlation of nifedipine oxidase activity with immunochemically determined P-450$_{NF}$ in human liver microsomes.** Microsomal samples prepared from 32 individual livers were assayed for nifedipine oxidase activity (using 50 pmol of total P-450, incubation time 10 min, substrate concentration, 0.20 mM) and for P-450$_{NF}$ using immunoblotting: 10 μg of microsomal protein were electrophoresed in each well along with 0.1 μg of equine liver alcohol dehydrogenase (Steward *et al.*, 1985). Each gel also contained lanes with 0.4, 1.0, 2.0, 5.0, and 10.0 pmol of purified P-450$_{NF}$ (from sample HL92) and the same amount of alcohol dehydrogenase. The nitrocellulose sheets were treated with a 1:100 dilution of rabbit anti-P-450$_{NF}$ (and a 1:500 dilution of anti-alcohol dehydrogenase), and amounts of P-450$_{NF}$ were estimated using the ratio of peaks (obtained after densitometry) of P-450$_{NF}$ and alcohol dehydrogenase (Steward *et al.*, 1985). The *line* through the points was drawn using linear regression analysis.

Since a role for cytochrome b_5 in nifedipine oxidase activity could also be demonstrated (*vide supra*), we also considered the effects of variation of this enzyme as well. Among 20 individual samples examined the specific content of cytochrome b_5 (estimated spectrally) varied from 0.25–1.19 nmol (mg protein)$^{-1}$ (mean 0.82 ± 0.24 (S.D.)). When nifedipine oxidase activity was compared to the amount of cytochrome b_5 in these samples, the *r* value was only 0.25 (not significant, $p > 0.10$).

DISCUSSION

In this report we have identified and characterized the P-450 isozymes involved in the oxidation of the drug nifedipine. Two rat P-450s, designated as P-450$_{UT-A}$ and P-450$_{PCN-E}$, were identified as having significant roles in the reaction. The human liver enzyme active in nifedipine oxidation, designated P-450$_{NF}$, was purified on the basis of catalytic activity and found to be apparently indistinguishable from several preparations of human liver P-450 which had previously been isolated in this laboratory (Wang *et al.*, 1983). Immunochemical inhibition studies strongly suggest that this enzyme is responsible for most of the primary oxidation of nifedipine, with the immunochemically determined levels of P-450$_{NF}$ being well correlated with nifedipine oxidase activity in human liver microsomes.

In rats, evidence for the roles of P-450$_{UT-A}$ and P-450$_{PCN-E}$ in nifedipine oxidation comes from both reconstitution and immunoinhibition studies. The 20-fold sex difference in untreated rats is consistent with the demonstration that in adult animals both of the isozymes mentioned are male specific (Waxman *et al.*, 1985). P-450$_{PCN-E}$ can be induced in males or females with pregnenolone 16α-carbonitrile, phenobarbital, dexamethasone, isosafrole, or triacetyloleandomycin (Guengerich *et al.*, 1982a; Heuman *et al.*, 1982; Wrighton *et al.*, 1985; Waxman *et al.*, 1985). P-450$_{UT-A}$ levels are decreased by the above compounds and particularly by β-naphthoflavone and other chemicals which induce P-450$_{\beta NF-B}$ (Dannan *et al.*, 1983; Waxman, 1984). The rat P-450$_{PCN-E}$ activity may have some relevance to the human situation, but caution is advised; the P-450$_{UT-A}$ activity is probably not useful in making comparisons to humans.

In humans evidence for the role of P-450$_{NF}$ in nifedipine oxidation comes from reconstitution and immunochemical inhibition studies. The correlation between catalytic activity and P-450$_{NF}$ levels also supports this conclusion. The question arises as to the identity and number of P-450s in humans which cross-react immunochemically with P-450$_{NF}$. Anti-P-450$_{NF}$ reacted with a minor protein(s) which was resolved using DEAE-cellulose but did not exhibit nifedipine oxidase activity (Fig. 2*B*). Furthermore, in previous work human liver P-450s that appeared to be immunochemically similar to each other could be separated chromatographically (Wang *et al.*, 1983). Recently Watkins *et al.* (1985) isolated a P-450 from human liver on the basis of its strong cross-reactivity with what appears to be rat P-450$_{PCN-E}$ or a homolog (catalytic activity was not reported). At this point we do not know the extent of microheterogeneity in the structure of P-450$_{NF}$ and related enzymes. The possibility certainly exists that this is a multigene family, but no further evidence is available.

Human P-450$_{NF}$ appears to share immunochemical homology (Figs. 6 and 11) as well as catalytic activity with rat P-450$_{PCN-E}$, insofar as P-450$_{PCN-E}$ contributes partially to nifedipine oxidation in rat liver microsomes. The regulation of P-450$_{NF}$ and P-450$_{PCN-E}$ is, however, quite distinct. Thus, P-450$_{PCN-E}$ is a male-specific enzyme in untreated adult rats, although present in young rats of both sexes before puberty. Neonatal gonadectomy and hormone replacement can be used to alter the patterns of P-450$_{PCN-E}$ expression in rats, as can xenobiotic administration (Waxman *et al.*, 1985). In humans no sex or age dependence of nifedipine metabolism has been observed (Kleinbloesem *et al.*, 1984). Interestingly, the liver sample which had the highest levels of both nifedipine oxidase activity *and* immunochemically detectable P-450$_{NF}$ (detected with both rabbit anti-P-450$_{NF}$ *and* the monoclonal antibody) (Fig. 14) was obtained from a patient who had been adminis-

tered dexamethasone.[7] This observation suggests that the enzyme may be inducible (*e.g.* by glucocorticoids), but comparison with the liver before treatment is not possible and conclusions regarding "induction" should be considered hypothetical. The correlation between nifedipine oxidase activity and the immunochemically determined levels of P-450$_{NF}$ suggests that the amount of the enzyme may be important in determining the catalytic activity. If this is the case, then the locus in determining whether a person is a phenotypic deficient metabolizer may be at the level of factors involved in transcription rather than the structural gene. Further experimentation will be required to address these hypotheses, but we feel that we have established a basis for such studies.

Various correlation, reconstitution, and immunochemical inhibition data support the view that P-450$_{NF}$ is a major enzyme involved in human liver nifedipine oxidation, aldrin epoxidation, 17β-estradiol 2- and 4-hydroxylation, and the 6β-hydroxylation of testosterone. In our previous studies we found that human liver microsomal *d*-benzphetamine demethylase activity was also inhibited by anti-human P-450$_3$, which now has been shown to recognize P-450$_{NF}$ (Wang *et al.*, 1983). This result has been confirmed with anti-P-450$_{NF}$ in the present study (data not shown). Thus, P-450$_{NF}$ or a closely related variant also appears to be the major isozyme responsible for this activity in human liver. The 6β-hydroxylation pathway appears to be the major route of oxidative hepatic metabolism of testosterone in humans of either sex (Kremers *et al.*, 1981). If a significant fraction of humans have an *in vivo* deficiency of this activity, the implications are unknown. Fishman *et al.* (1980) reported that a sizable fraction of the human population was unable to hydroxylate 17β-estradiol *in vivo*, and a sex difference was noted. It is interesting that the fraction of individuals deficient in estrogen metabolism is similar to the fraction deficient in *in vivo* nifedipine oxidation (Kleinbloesem *et al.*, 1984), although in the nifedipine study no sex difference was observed. Whether the enzyme we have characterized is involved in both nifedipine oxidation and 17β-estradiol hydroxylation *in vivo* is unknown and will require further study.

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[7] Kindly provided by Dr. P. S. Guzelian, Medical College of Virginia, Richmond, VA.

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