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Inhibition of Oxidative Drug Metabolism by Orphenadrine: *In Vitro* and *In Vivo* Evidence for Isozyme-Specific Complexation of Cytochrome P-450 and Inhibition Kinetics

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SUMMARY

The anti-parkinsonian agent orphenadrine has been shown to form an *in vitro* metabolic intermediate (MI) complex in hepatic microsomes isolated from phenobarbital (PB)-treated rats. The present study was undertaken to assess the cytochrome P-450 isozyme specificity of inhibition and MI complexation. Spectral studies with untreated and PB-induced rat hepatic microsomes confirmed earlier reports on the selectivity of P-450 complexation by orphenadrine; MI complex formation was only observed with PB-induced microsomes. Inhibition studies with the P-450 substrates androst-4-ene-3,17-dione (androstenedione) and 7-pentoxyresorufin revealed selective inhibition of P-450 PB-B/D-associated monooxygenase activity. Thus, in microsomes from untreated male rats, orphenadrine failed to significantly inhibit (<50% inhibition up to a concentration of 300 μM) any of the major pathways of P-450-associated androstenedione metabolism. Preincubation of these microsomal fractions with orphenadrine and NADPH was not associated with increased inhibition of androstenedione metabolism. However, in PB-induced microsomes, P-450 PB-B/D-specific androstenedione 16β-hydroxylase activity was significantly and selectively inhibited (IC_{50} = 90 μM). Preincubation of orphenadrine with NADPH-supplemented PB-induced microsomes for 2, 4, or 8 min before androstenedione addition resulted in increased inhibition toward 16β-hydroxylase activity, lowering the observed IC_{50} to 6.6, 0.47, and 0.06 μM, respectively. Preincubation did not affect the selectivity of inhibition. In the absence of preincubation, orphenadrine appeared to be a potent mixed (competitive/noncompetitive)-type inhibitor of P-450 PB-B/D-associated pentoxyresorufin *O*-depentylation (K_i = 3.8 μM). Preincubation of orphenadrine with NADPH-supplemented microsomal fractions for 4 min resulted in a 30-fold lowering of the apparent inhibitor constant (K_i = 0.13 μM) and a change in the apparent inhibition kinetics to noncompetitive. Treatment of rats with orphenadrine (75 mg/kg/day intraperitoneally for 3 days) was associated with a 2-fold induction of total hepatic P-450, a 5- and 2.4-fold induction of androstenedione 16β- and 6β-hydroxylase activity, respectively, and formation of an orphenadrine-P-450 MI complex. Western blots of orphenadrine-induced microsomes revealed a 20-fold increase in P-450 PB-B/D-immunoreactive protein. Dissociation of the P-450 MI complex by treatment with potassium ferricyanide (100 μM) during preparation of orphenadrine-induced microsomes was associated with a 12, 15, and 13% increase in P-450 and androstenedione 16α- and 16β-hydroxylation activity, respectively. Similarly, potassium ferricyanide treatment of microsomes isolated from rats treated with PB and dosed with orphenadrine was associated with a 14, 38, 28, and 16% increase in P-450, pentoxyresorufin *O*-dependentylase, and androstenedione 16α- and 16β-hydroxylase activities, respectively. The data presented here are consistent with the assertion that orphenadrine is a potent and selective inhibitor of P-450 PB-B/D-associated monooxygenase activity *in vitro* and an inducer of the same isozyme (P-450 PB-B/D) *in vivo*. MI complexation *in vivo* also appears to be selective for this isozyme. Selective complexation/inhibition of a constitutive human P-450 analogous to rat form PB-B/D may explain the observed increase in elimination half-life of orphenadrine in humans, following a multiple-dose regimen.

The hepatic microsomal cytochrome P-450 (P-450)[1] monooxygenase system plays a central role in the oxidative metabolism of a wide range of both endogenous and exogenous compounds (1–3). It is now evident that the wide substrate specificity of this system is due, in part, to the large number of P-450 isozymes found in hepatic tissue (4). Each P-450 isozyme exhibits a characteristic range of activities in the oxidation of various substrates (5, 6).

The isozymic nature of the hepatic P-450 system renders it susceptible to inhibition by a wide array of chemicals both *in*

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[1] According to the recently recommended nomenclature for cytochrome P-450 (33), rat hepatic cytochromes P-450 UT-A, PB-B/D, PCN-E, and UT-F are encoded by genes IIC 11, IIB 1/2, IIIA 1/2, and IIA 1, respectively.

ABBREVIATIONS: MI, metabolic intermediate; PB, phenobarbital; FCN, potassium ferricyanide; androstenedione, androst-4-ene-3,17-dione; P-450, cytochrome P-450; IgG, immunoglobulin G; BSA, bovine serum albumin; TBS, Tris-buffered saline.

vitro and after *in vivo* administration to experimental animals (7). Of particular interest are those compounds that generate reactive metabolites that interact with the active center of P-450 and result in the formation of MI complexes. The alkylamine agents, typified by SKF 525-A, macrolide antibiotics, and orphenadrine, elicit MI complexes with P-450 and effectively sequester P-450 in a nonfunctional state. Dissociation of these nonfunctional MI complexes is achieved after *in vitro* oxidation of the ferro-heme complex with an agent such as FCN (8, 9). These MI complexes exhibit characteristic maxima in the range 450–460 nm of the absorbance difference spectrum (10).

The antiparkinsonian agent orphenadrine (Fig. 1) has been shown to form both *in vivo* and *in vitro* P-450-MI complexes in rats (11–13). MI complex formation has been offered as a possible cause for the observed aberrant pharmacokinetics following multiple doses of orphenadrine in humans (11, 14). However, in rats P-450-MI complex formation appeared to be dependent on pretreatment of animals with PB. In the absence of PB induction, little or no complex formation was observed (11–13). These results suggest that orphenadrine P-450-MI complex formation may well be isozyme specific. Certainly, the available evidence is consistent with the assertion that one or more members of the PB-inducible subfamily of P-450 isozymes is/are involved in orphenadrine MI complex formation.

The present study was designed to investigate the P-450 isozyme specificity of orphenadrine MI complex formation in relation to inhibition of oxidase activity. To aid in identification of the P-450 isozymes involved in orphenadrine MI complexation, the regioselective hydroxylation of androstenedione was employed because it is now well established that, in hepatic microsomes isolated from untreated male rats, the formation of the 6β-, 7α-, 16α-, and 16β-hydroxyandrostenedione metabolites are catalyzed specifically by P-450 isozymes [following the nomenclature of Guengerich *et al.* (1)] PCN-E, UT-F, UT-A, and PB-B/D, respectively.

Materials and Methods

Chemicals. Orphenadrine hydrochloride was a generous gift from Riker Laboratories Aust. Pty. Ltd. Pentoxyresorufin was purchased from Pierce Chemicals (Rockford, IL) resorufin was obtained from Aldrich Chemical Company (St. Louis, MO), and PB was purchased from BDH Aust. Ltd. Solvents and miscellaneous chemicals were purchased from Ajax Chemicals (Sydney, Australia) and were of the highest grade commercially available.

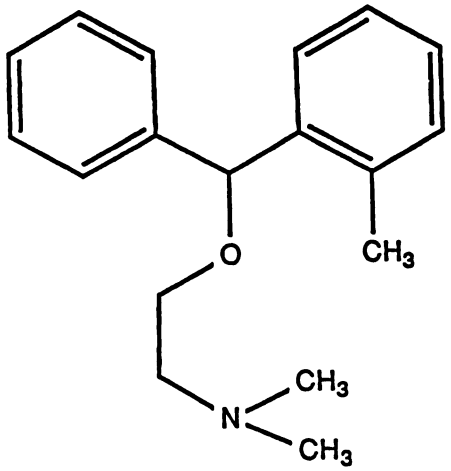

Fig. 1. Structure of orphenadrine.

[4-^{14}C]Androstenedione (specific activity 59 mCi/mmol) was obtained from Amersham Australia (Sydney, NSW). Testosterone, unlabeled androstenedione, and its 6β- and 16α-hydroxy metabolites, as well as all biochemicals, were purchased from Sigma Chemical Co. (St. Louis, MO).

7α-Hydroxyandrostenedione was obtained from Professor D. N. Kirk of the MRC Steroid Reference Collection, Queen Mary's College (London, United Kingdom). 16β-Hydroxyandrostenedione was prepared enzymatically by the action of 3β-hydroxysteroid dehydrogenase (Sigma) on 3β,16β-dihydroxyandrost-5-ene-17-one (MRC Collection) as described by Talalay and Dobson (15).

Animals. Male Wistar rats (250–300 g) were obtained from the animal house of the Institute of Clinical Pathology and Medical Research at Westmead Hospital. Animals were held in wire cages under conditions of constant temperature (22°), lighting (12-hr dark-light cycle), and humidity. In some experiments, male rats were treated for 3 consecutive days, intraperitoneally, with either orphenadrine hydrochloride (75 mg/kg/day) or phenobarbital (80 mg/kg/day) dissolved in saline. Controls received saline alone. In these experiments rats were sacrificed 24 hr after the final injection. In one experiment rats were treated with phenobarbital (80 mg/kg/day) for 3 days and then administered a single intraperitoneal dose of orphenadrine (40 mg/kg) 14 hr before sacrifice. Microsomes were prepared as previously described (7).

Dissociation of the P-450-orphenadrine metabolite complex formed *in vivo*. Dissociation of the orphenadrine metabolite from its complex with microsomal P-450 was effected during the preparation of microsomal fractions by differential ultracentrifugation. After the first ultracentrifugation step (1 hr at 105,000 × *g*), microsomal pellets from either orphenadrine-induced or untreated rat liver were resuspended in microsomal preparation buffer (0.25 M sucrose, containing 1 mM EDTA and 10 mM potassium phosphate, pH 7.4), and divided equally between two ultracentrifuge tubes. FCN was added to one half of each resuspended microsomal pellet to a final concentration of 100 μM. Thirty minutes later the microsomal pellets were reisolated at 105,000 × *g* before a final wash step to remove any residual FCN. Microsomes were stored at −70° as suspensions in 20% glycerol containing 1 mM EDTA and 50 mM potassium phosphate, pH 7.4, and were used within 3 days of preparation. An analogous experiment, in which hepatic microsomes were isolated from PB-treated rats administered orphenadrine 14 hr before sacrifice, was also conducted.

Absorbance difference spectroscopy. The difference spectral interaction between ferric P-450 and orphenadrine was measured *in vitro* at 37° in an Aminco-Chance DW-2a spectrophotometer using 1-cm cuvettes containing 1-ml aliquots of microsomal suspensions from PB-treated or untreated rats (2 mg of microsomal protein/ml) in potassium phosphate buffer (0.1 M, pH 7.4). Orphenadrine was added to the sample cuvette in microliter quantities of buffer and the difference spectra were recorded between 380 and 500 nm; an equal volume of buffer was added to the reference cuvette. Spectral dissociation constants (K_D) and maximal spectral changes (ΔA_{max}) were determined from double-reciprocal plots of the data according to the method of Murray and Ryan (16). MI complex formation was monitored at 37° in an Aminco-Chance DW-2a spectrophotometer (17). Metabolism was initiated by addition of NADPH (final concentration, 1 mM) to both reference and sample cuvettes. Repetitive scans over the 380–500 nm region of the spectrum were performed for at least 15 min after NADPH addition.

Cytochrome P-450 was measured according to the method of Omura and Sato (18) with an Aminco-Chance DW-2a spectrophotometer using an extinction coefficient of 91 $mM^{-1}\ cm^{-1}$ for the ferrous P-450-carbonyl spectral complex. In some experiments, dissociation of the ferrous-P-450-orphenadrine metabolite complex was followed spectrophotometrically (Aminco-Chance DW-2a) after the addition of FCN (50 μM) to orphenadrine-induced rat hepatic microsomes (2 mg/ml).

Microsomal monooxygenase activities. Microsomal androstenedione hydroxylase activity was assayed essentially as described previously (7). Each incubation (4.0 ml final volume) contained 3.0 mg of

microsomal protein, an NADPH-generating system (4 mM glucose 6-phosphate, 1 mM NADP, and 1 unit of glucose 6-phosphate dehydrogenase) and androstenedione (final concentration, 175 μM). Reactions were initiated by addition of the generating system and were terminated by the addition of 2 ml of 5.5% zinc sulfate. Reaction times were 10 min for control and 5 min for PB-induced microsomes. The chloroform extract of the reaction supernatant was applied to thin layer chromatography plates (silica gel 60, F_{254} type, 20 × 20 cm × 0.25 mm thick; activated at 100° for 15 min before use; E. Merck, Darmstadt, FRG). Plates were developed twice in the solvent system ($CHCl_3$/ethyl acetate, 1:2, v/v) as described by Waxman *et al.* (19). Zones corresponding to hydroxylated androstenedione standards were visualized under UV light and scraped into vials for scintillation counting.

Orphenadrine was introduced into the reaction mixture in 50 μl of buffer. In some cases, NADPH-fortified microsomes were preincubated with different concentrations of orphenadrine for 2, 4, or 8 min before addition of the substrate, androstenedione. Plots of log inhibitor concentration versus percent of control activity were constructed from mean percent inhibition data at eight different inhibitor (orphenadrine) concentrations. Each point was the mean of at least two individual estimates from separate incubations.

Pentoxyresorufin *O*-dependentylase activity was measured in PB-induced microsomes as described by Lubet *et al.* (20). Each incubation contained 0.2 mg of microsomal protein in a final volume of 2 ml. The reaction was initiated by the addition of 10 μl of NADPH to give a final concentration of 1 mM. The substrate (pentoxyresorufin) concentration was varied between 1 and 10 μM and the inhibitor (orphenadrine) concentration between 0 and 25 μM. In some experiments, NADPH-fortified microsomes were preincubated with orphenadrine for 4 min before initiation of the reaction by the addition of the substrate, pentoxyresorufin.

Purification of the PB-inducible P-450 PB-B. P-450 PB-B was isolated from cholate-solubilized male rat hepatic microsomes essentially by the method of Guengerich and Martin (21). An additional purification step on hydroxylapatite (Bio-gel HT; Bio-Rad, Sydney, Australia) was performed at 4°, as described by Waxman *et al.* (22). P-450 PB-B eluted after the column was washed with 90 mM potassium phosphate, and final clean-up was performed on CM-Trisacryl M (LKB, Bromma, Sweden) and DEAE-Sephacel (Pharmacia, Sydney, Australia) at room temperature. Detergent removal was achieved using a small hydroxylapatite column. The specific content of the final preparation was 13 nmol of P-450/mg of protein.

Preparation of anti-P-450 PB-B IgG. The raising of antisera to P-450s was as described previously (7). IgG was isolated from rabbit serum and recycled for 15 hr through a column of solubilized hepatic microsomes from untreated male rats that had been coupled to CNBr-activated Sepharose 4B (19). The rabbit anti-rat anti-P-450 PB-B IgG recovered from this procedure was investigated for its inhibitory properties toward a number of P-450-mediated reactions. In these experiments, the IgG was preincubated with an appropriate microsomal fraction at room temperature for 40 min (5 mg of IgG/mg of microsomal protein). 7-Pentoxyresorufin *O*-dependentylase and androstenedione 16β-hydroxylase activities in microsomes from PB-induced rats were both decreased by the IgG, to 21% and 32% of the respective uninhibited controls. In contrast, androstenedione 6β- and 7α-hydroxylase activities in these microsomes and 7-ethoxyresorufin *O*-deethylase activity in microsomes from β-naphthoflavone-treated rats were uninhibited.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Hepatic microsomal fractions from control (66 μg of protein/lane) and orphenadrine-treated (8.33 μg of protein/lane) rats were prepared for electrophoresis by incubation with 2% sodium dodecyl sulfate and 5% 2-mercaptoethanol at 100° for 5 min. Electrophoresis was conducted according to the method of Laemmli (23), modified by doubling the concentrations of Tris and glycine in all buffers and gels. Lanes containing known amounts of pure P-450 PB-B (0.1–2.0 μg of protein) were also electrophoresed.

Transfer of proteins to nitrocellulose and immunochemical detection. After electrophoresis, proteins were transferred to nitrocellulose sheets by the method of Towbin *et al.* (24). The sheets were then shaken in TBS (50 mM Tris, 200 mM NaCl, pH 7.4) for 30 min, followed by an overnight incubation in 3% BSA in TBS. The next step involved incubation of the nitrocellulose sheets with anti-PB-B IgG (1:500 dilution of 28.6 mg of protein/ml) for 2 hr, followed by five washes in TBS (5 min each) and then a 10-min wash in TBS containing 3% BSA. Sheets were incubated with a combination of ^{125}I-labeled and peroxidase-labeled donkey anti-rabbit IgG (Amersham, UK; 2×10^5 cpm/ml; dilution as recommended by the supplier) in TBS containing 3% BSA. Blots were visualized with 4-chloro-1-naphthol/hydrogen peroxide, excised, and subjected to γ counting (LKB Multigamma 1260). Radioactivity was found to be proportional to the amount of antigen used in this study for the construction of standard curves.

Protein assay. Microsomal protein concentration was estimated using the modified Lowry method of Chaykin (25).

Graphical methods. Inhibitory data from catalytic experiments were plotted according to the method of Lineweaver and Burk (26) and, in some instances, the method of Dixon (27). Lines of best fit were calculated by linear regression.

Results

Interaction of orphenadrine with hepatic microsomes *in vitro*. The incubation of orphenadrine with oxidized hepatic P-450 resulted in a type I spectral change (low to high spin transition) in both untreated and PB-induced microsomes (data not shown). The extent of interaction of orphenadrine with P-450 (ΔA_{max}) appeared small in both types of microsomes. However, PB induction was associated with an approximate 100-fold increase in the apparent spectral dissociation constant (K_D) of orphenadrine, compared with untreated microsomes (Table 1).

MI complex formation was not observed in NADPH-fortified microsomes isolated from untreated male rats but incubation of orphenadrine with NADPH-fortified microsomes isolated from PB-treated rats resulted in the formation of a MI complex with an absorbance maximum of 456 nm and was associated with the loss of quantifiable P-450. Thus, incubation of orphenadrine (30 μM) with NADPH and PB-induced microsomes at 37° resulted in 10–15% sequestration of P-450 after 8–10 min. *In vitro* complexation with orphenadrine was apparently complete after this time period, because incubation for longer periods did not result in either increased P-450 sequestration or 456 nm complex formation (data not shown). Complex formation appeared to be specific for phenobarbital induced microsomes, because incubation of orphenadrine with clofibrate-, isosafrole-, β-naphthoflavone-, isoniazid-, or pregnenolone 16α-carbonitrile-induced microsomes, supplemented with NADPH, failed to result in significant formation of a spectrally detectable MI complex (data not shown).

***In vitro* inhibition of androstenedione hydroxylation**

TABLE 1

Parameters of binding interactions of orphenadrine in hepatic microsomes from untreated and PB-treated male rats

Values are means ± standard deviations of three individual titrations.

Microsomes	Binding in oxidized microsomes		Binding in NADPH-fortified microsomes, MI complex $\Delta A_{456-490}$
	K_D	ΔA_{max}	
	μM	absorbance unit/nmol of P-450	absorbance unit/nmol of P-450
Untreated	0.12 ± 0.01	$1.2 \pm 0.1 \times 10^{-2}$	—
PB-induced	15.0 ± 3.0	$0.9 \pm 0.1 \times 10^{-2}$	$0.9 \pm 0.1 \times 10^{-2}$

by orphenadrine. When incubated with microsomes from untreated male rats, orphenadrine (in the concentration range 1–300 μM) failed to significantly inhibit any of the major pathways associated with hepatic P-450 androstenedione metabolism, although 40% inhibition of the 16β-hydroxylase pathway was observed (Table 2). IC_{50} values could not be estimated for the inhibition of androstenedione hydroxylation in control microsomes due to the lack of potency of orphenadrine. However, as shown in Table 2, androstenedione 16β-hydroxylation in PB-induced microsomes was selectively inhibited by orphenadrine (IC_{50} = 90 μM). Preincubation of orphenadrine for 2, 4, or 8 min with PB-induced microsomes was associated with dramatic decreases in the IC_{50} (increasing inhibitory potency) for androstenedione 16β-hydroxylation. Thus, after 2, 4, and 8 min of preincubation, there was an associated 14-, 188-, and 1500-fold decrease in the IC_{50} of orphenadrine toward androstenedione 16β-hydroxylation, respectively. The potency of the inhibition resulting from 8 min of preincubation can be gauged from the fact that the concentration of orphenadrine required to effect 50% inhibition of androstenedione 16β-hydroxylation is some 3000 times less than the substrate concentration used in the assay (175 μM). The specificity of inhibition was unaffected by preincubation. Preincubation of control microsomal fractions with orphenadrine and NADPH did not alter the specificity of inhibition produced by the alkylamine drug. Considered together these observations are consistent with the selective involvement of P-450 PB-B in the inhibition/complexation phenomena.

Kinetics of monooxygenase inhibition by orphenadrine. The kinetics of orphenadrine inhibition in PB-induced microsomes was examined in further studies, with and without preincubation, using pentoxyresorufin as the substrate. Pentoxyresorufin *O*-dependentylation reflects the activity of the same form of P-450 involved in androstenedione 16β-hydroxylation (20) but, unlike the steroid hydroxylase assay, has the advantage that the time-dependent formation of product may be followed directly.

Lineweaver-Burk (Fig. 2) and Dixon (Fig. 3) analysis of inhibition data indicated that, in the absence of preincubation with NADPH, orphenadrine exerted mixed-type inhibition of pentoxyresorufin *O*-dependentylase activity in PB-induced microsomes. Because the family of lines of the Lineweaver-Burk reciprocal plot intersect above the $[S]^{-1}$ axis to the left of the V^{-1} axis and because the Dixon plot is linear, the inhibition is described more precisely as 'intersecting linear noncompetitive' (28). Such inhibition is considered to result from a mixture of partial competitive and pure noncompetitive inhibition (28). The apparent K_i for orphenadrine in this system was estimated graphically from the Dixon plot and was found to be 3.8 μM (Table 3).

Lineweaver-Burk (Fig. 4) analysis of the inhibition of pent-

TABLE 2

Inhibition of androstenedione hydroxylase activity in hepatic microsomes from untreated and PB-treated rats by orphenadrine

IC_{50} values were determined in triplicate from plots of log (inhibitor) concentration versus percent inhibition, using eight concentrations of orphenadrine. In all cases $r > 0.96$. Control activities (nmol/min/mg of protein) for androstenedione 16α-, 16β-, 6β-, and 7α-hydroxylation were 2.7 ± 0.4, 8.1 ± 0.3, 7.4 ± 0.2, and 0.95 ± 0.02 (PB-treated rat liver) and 2.22 ± 0.03, 0.38 ± 0.08, 2.8 ± 0.10, and 0.5 ± 0.07 (untreated rat liver).

Microsomes	Pre-incubation time (min)	IC_{50} versus androstenedione hydroxylase activity			
		16α	16β	6β	7α
			μM		
Untreated	0	—[a]	(48)[b]	—	—
	10	—	(43)[b]	—	—
Phenobarbital-treated	0	—	90	—	—
	2	—	6.6	—	—
	4	—	0.48	—	—
	8	—	0.06	—	—

[a] Not determined due to insufficient inhibition at 300 μM orphenadrine.
[b] Percent inhibition of control activity observed at 300 μM orphenadrine.

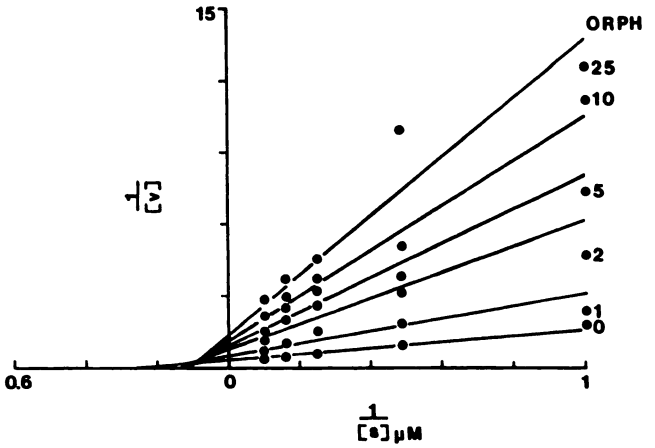

Fig. 2. Lineweaver-Burk plots of the inhibition of pentoxyresorufin *O*-dependentylase activity, in the absence of preincubation, by orphenadrine (*ORPH*) (concentration range, 0–25 μM) in PB-induced microsomes.

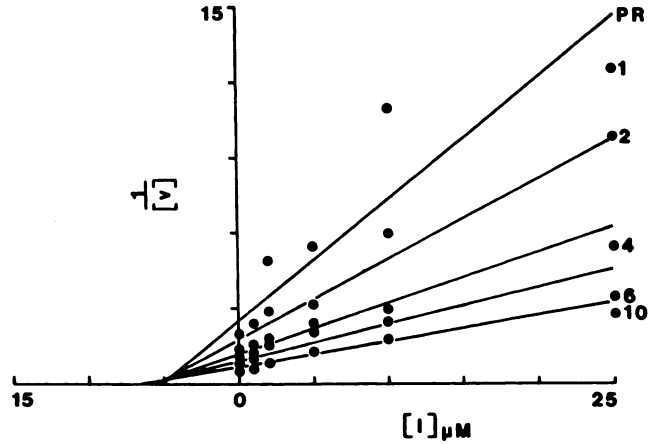

Fig. 3. Dixon plots (inhibitor concentration versus reciprocal reaction velocity) of the inhibition of pentoxyresorufin *O*-dependentylase activity by orphenadrine in PB-induced microsomes. *PR*, pentoxyresorufin (concentration range, 1–10 μM).

TABLE 3

Apparent kinetic parameters of pentoxyresorufin O-dependentylase activity and its inhibition by orphenadrine in PB-induced hepatic microsomes

Pentoxyresorufin *O*-dependentylase activity	
K_s (μM)	3.5
V_{max} (nmol of resorufin/min/mg of protein)	2.4
Inhibition by orphenadrine	
No preincubation	
K_i (μM)	3.8
α	2.0
4-min preincubation	
K_i (μM)	0.13

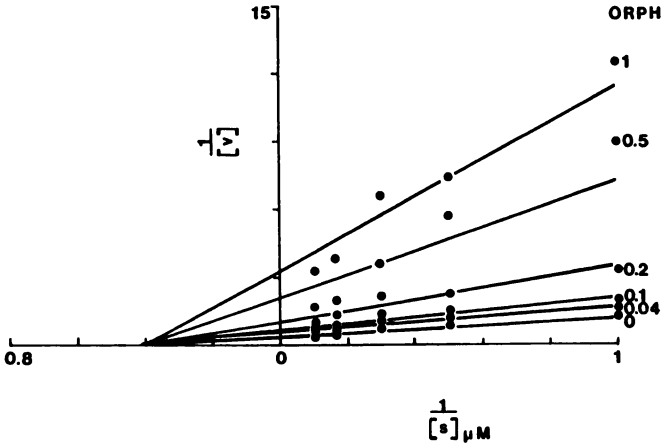

Fig. 4. Lineweaver-Burk plots of the inhibition of pentoxyresorufin *O*-dependentylase activity following 4 min of preincubation of orphenadrine (*ORPH*) (concentration range, 0–1 μM) with NADPH-supplemented, PB-induced microsomes.

oxyresorufin *O*-dependentylation in PB-induced microsomes by orphenadrine, following 4 min of preincubation with NADPH, revealed apparent noncompetitive kinetics. Orphenadrine preincubation was also associated with a dramatic 30-fold lowering of apparent K_i to 0.13 μM (Table 3). The observed change in inhibition kinetics associated with preincubation, from a mixed-type with a component of competitive inhibition to apparent noncompetitive, is consistent with the progressive formation of an inhibitory metabolite resulting in an enzyme-substrate-inhibitor complex that is nonproductive. Noncompetitive kinetics suggest that the inhibitor binds in a region other than the substrate binding site. In the case of P-450, adduction of reactive metabolites to the prosthetic heme has previously been associated with noncompetitive monooxygenase inhibition (9, 10).

Effects of administration of orphenadrine on male rat hepatic microsomal cytochrome P-450 and steroid hydroxylase activities. As shown in Table 4, administration of orphenadrine to male rats was associated with an apparent 2.2-fold increase in total hepatic microsomal P-450. Similarly, androstenedione 16β- and 6β-hydroxylase activities were induced approximately 5- and 2.4-fold, respectively, compared with controls. Western blots (Table 5) of hepatic microsomal fractions isolated from orphenadrine-treated rats revealed a 20-fold increase in P-450 PB-B-immunoreactive protein, a finding consistent with elevated pentoxyresorufin *O*-dependentylase and androstenedione 16β-hydroxylase activities in these microsomes. In contrast, however, androstenedione 16α-hydroxylation was significantly decreased to approximately 52% of control, whereas 7α-hydroxylase activity was unchanged (Table 4). A number of other alkylamine agents are known to elicit P-450 complexation *in vivo* and the resultant complex has been shown to be dissociated by FCN *in vitro*. Thus, a portion of each microsomal fraction in the present study was incubated with FCN during isolation; the FCN was removed in a later washing procedure. Microsomes treated in this manner showed a further increase in apparent P-450 content and the rate of androstenedione hydroxylation. FCN treatment of orphenadrine-induced microsomes was associated with a 12% increase in hepatic P-450 over levels observed in 'undissociated' microsomes and selective increases in both androstenedione 16α- and 16β-hydroxylation (150% and 130% of undissociated microsomal activities, respectively). Androstenedione 6β- and 7α-hydroxylation pathways were unaffected. Similarly, FCN treatment of microsomes isolated from rats treated with PB, and administered a single intraperitoneal dose of orphenadrine 14 hr before sacrifice, was associated with increases in cytochrome P-450 content and monooxygenase activity (Table 6). Thus, P-450 content and pentoxyresorufin *O*-dependentylation and androstenedione 16α- and 16β-hydroxylase activities were found to be 114, 138, 128, and 116% of the respective undissociated controls. Again, androstenedione 6β- and 7α-hydroxylase activities were unchanged following FCN treatment. From these findings, it would appear that orphenadrine has the capacity to induce P-450 and to generate an MI complex with P-450 PB-B *in vivo*. The available evidence is also consistent with the assertion that the *in vivo* complexation of P-450 by orphenadrine is isozyme selective.

When monitored spectrophotometrically, *in vitro* addition of FCN to hepatic microsomes isolated from orphenadrine-treated rats resulted in the appearance of a trough at 456 nm (Fig. 5). The appearance of this trough provides further evidence that a P-450 MI complex is generated *in vivo* following orphenadrine administration.

Discussion

The data presented here strongly suggest that orphenadrine is a potent and selective *in vitro* inhibitor of P-450 PB-B/D associated monooxygenase activity; this inhibition has been shown to be associated with MI complex formation. Thus, little inhibition of P-450 PB-B/D-catalyzed androstenedione 16β-hydroxylation was observed in microsomes isolated from untreated rats (in which orphenadrine P-450-MI complexes were

TABLE 4

Effects of FCN treatment on cytochrome P-450 content and androstenedione hydroxylase activity in control and orphenadrine-induced male rat liver microsomes

Values are means ± standard errors from five experiments.

Microsomes	FCN dissociation[a]	Apparent P-450 content	Androstenedione hydroxylase activity			
			16α	16β	6β	7α
		nmol/mg of protein	*nmol/min/mg of protein*			
Control	−	0.70 ± 0.03	3.05 ± 0.27	0.35 ± 0.02	1.70 ± 0.35	0.39 ± 0.04
	+	0.74 ± 0.04	3.10 ± 0.27	0.33 ± 0.03	1.50 ± 0.34	0.34 ± 0.02
Orphenadrine-induced[b]	−	1.5 ± 0.08[c]	1.62 ± 0.23[c]	1.63 ± 0.23[c]	4.12 ± 0.40[c]	0.48 ± 0.03
	+	1.8 ± 0.12[d]	2.43 ± 0.41[d]	2.05 ± 0.20[c,d]	3.76 ± 0.46[c]	0.48 ± 0.06

[a] Microsomes were incubated with 100 μM FCN and reisolated as described in Materials and Methods.
[b] Rats were treated with orphenadrine-HCl (75 mg/kg/day, intraperitoneally, for 3 days).
[c] Significantly different from appropriate control (analysis of variance; $p < 0.05$).
[d] Significantly different from non-FCN-treated microsomes (Student's paired *t* test; $p < 0.05$).

TABLE 5

Quantitation of immunoreactive P-450 PB-B/D protein in microsomes from control and orphenadrine-treated rats

Male rats were treated with orphenadrine-HCl for 3 days (75 mg/kg/day, intraperitoneally), with five in each group. Values are mean ± standard error.

Microsomes	P-450 PB-B/D-immunoreactive protein
	nmol of P-450 PB-B/D/mg of protein
Control	0.096 ± 0.02
Orphenadrine-induced	1.91 ± 0.14

not generated). In contrast, MI complex formation appeared to be quite extensive in microsomal fractions from PB-treated rats. Of particular interest is the present finding that increased inhibition of androstenedione 16β-hydroxylation occurred after preincubation of orphenadrine with PB-induced microsomes. As shown here, and in other studies (11), orphenadrine MI complex formation is time dependent and is essentially complete after 8 min in PB-induced microsomes. Inhibition of androstenedione 16β-hydroxylation was quite pronounced after 8 min of preincubation and was associated with a change in the kinetics of inhibition. Thus, in the absence of preincubation, inhibition of PB-B/D-specific pentoxyresorufin *O*-dependentylase activity by orphenadrine was mixed (partial competitive/pure noncompetitive) with an apparent K_i of 3.8 μM. Preincubation of orphenadrine with NADPH-supplemented microsomes from PB-induced rats for 4 min, however, resulted in a 30-fold lowering of apparent K_i and a change in kinetics to apparent noncompetitive inhibition. Such changes in inhibition kinetics for MI complex-forming agents following preincubation have been noted before (10, 29). Because apparent K_i is simply a measure of the affinity of an inhibitor for a particular enzyme, the substantial lowering of K_i following preincubation suggests the formation of an orphenadrine metabolite(s) with a much higher affinity for P-450 PB-B/D than the parent compound. The general scheme describing inhibition of pentoxyresorufin *O*-dependentylation by orphenadrine (without preincubation), in PB-induced microsomes, is shown in Fig. 6. In this scheme the variable α is the factor by which K_s and K_i are altered in the presence of inhibitor (orphenadrine) and substrate (pentoxyresorufin), respectively. Following this scheme, it is apparent that preincubation drives most of the enzyme to the nonproductive *ESI* form and results in a lowered k_p value.

Alkylamine P-450-MI complexation has previously been associated with quasi-irreversible inhibition (9). The kinetics of quasi-irreversible and reversible pure noncompetitive inhibition are similar because, in both types of inhibition, the enzyme-substrate-inhibitor complex is nonproductive, as revealed by the lowered apparent V_{max}. Thus, the observed change, from mixed to apparent noncompetitive inhibition is consistent with an increase in the formation of a quasi-irreversible orphenadrine P-450-MI complex during preincubation. The apparent noncompetitive nature of the inhibition also suggests that the orphenadrine metabolite binds or adducts to a region other than the P-450 substrate binding site. This region is most probably the heme prosthetic group, which is both vulnerable to inhibition and critical for monooxygenase activity (9). The pentoxyresorufin *O*-dependentylation kinetic data are, therefore, consistent with the interpretation that an orphenadrine P-450 MI-complex is associated with the observed *in vitro* increase in inhibition of monooxygenase activity following preincubation. It is likely, however, that other orphenadrine metabolites, distinct from those directly involved in MI complexation, may also inhibit P-450 PB-B-mediated activity. Thus, orphenadrine MI complexation may account for only a portion of the inhibition of pentoxyresorufin *O*-dependentylase and androstenedione 16β-hydroxylase activities observed *in vitro*.

An interesting finding from this study was that even at very high orphenadrine concentrations (300 μM), without preincubation, constitutive steroid 16β-hydroxylase activity was not markedly inhibited (<40%). Yet, the IC_{50} for 16β-hydroxylase activity in PB-treated rats was 90 μM. If the same forms of PB-inducible P-450 are responsible for catalysis of this oxidase activity in both PB-induced and control microsomes, then a similar susceptibility to inhibition by orphenadrine would have been anticipated. The observation that androstenedione 16β-hydroxylase activities in control microsomes appeared resistant to orphenadrine inhibition suggests that different P-450 isozymes, differing in susceptibility to orphenadrine inhibition, are responsible for the catalysis of this pathway in PB-induced and control microsomes. A recent paper by Wilson *et al.* (30) has shown that P-450b (PB-B) is expressed in very low amounts in the livers of untreated Wistar rats (the strain used in this study) but, following PB induction, this enzyme is expressed in high concentration. In controls, P-450e (PB-D) appears to be expressed in higher amounts than P-450b (PB-B). Consequently, in the strain of rat used in this study P-450 PB-D (P-450e) could be responsible for the steroid 16β-hydroxylase

TABLE 6

Effects of FCN treatment on cytochrome P-450 content, pentoxyresorufin *O*-dependentylase, and androstenedione hydroxylase activities in hepatic microsomes from PB-treated rats and PB-treated rats treated with orphenadrine

Microsomes	FCN dissociation[a]	Apparent P-450 content	PROD[b]	Androstenedione hydroxylase activity			
				16α	16β	6β	7α
		nmol/mg of protein	*nmol/min/mg of protein*	*nmol/min/mg of protein*			
PB-induced[c]	−	2.91 ± 0.18[d]	4.6 ± 0.64	3.92 ± 0.33	10.82 ± 0.02	10.85 ± 0.35	1.12 ± 0.04
	+	3.00 ± 0.03	5.0 ± 0.57	3.80 ± 0.13	10.01 ± 0.20	10.96 ± 0.52	1.13 ± 0.03
PB-induced + orphenadrine[e]	−	2.82 ± 0.05[f]	3.6 ± 0.30	3.83 ± 0.27	9.95 ± 0.86	11.04 ± 0.94	1.26 ± 0.03
	+	3.22 ± 0.08[g]	5.0 ± 0.80[g]	4.93 ± 0.60[g]	11.55 ± 1.70[g]	11.25 ± 0.47	1.25 ± 0.01

[a] Microsomes were incubated with 100 μM FCN and reisolated as described in Materials and Methods.
[b] PROD, pentoxyresorufin *O*-dependentylase activity.
[c] Rats were treated with PB for 3 days (80 mg/kg/day, intraperitoneally).
[d] Mean ± SE; $n = 3$.
[e] Rats were treated with PB for 3 days (80 mg/kg/day, intraperitoneally). Rats were administered orphenadrine (40 mg/kg intraperitoneally) 14 hr before sacrifice.
[f] Mean ± SE; $n = 4$.
[g] Significantly different from non-FCN-treated microsomes (Student's paired t test; $p < 0.05$).

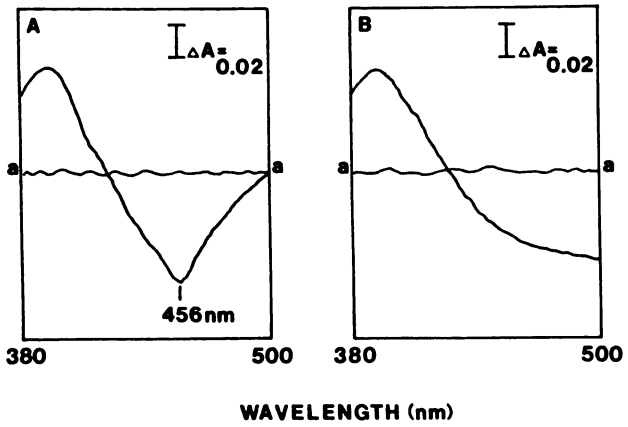

Fig. 5. Absorbance difference spectrum produced by the addition of 50 μM FCN to hepatic microsomes from orphenadrine-treated (A) and control (B) rats. Trace *a* is a baseline of zero light absorbance.

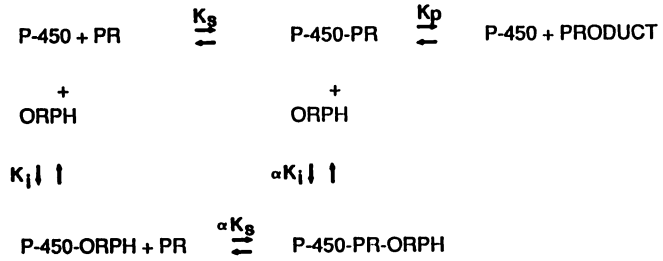

Fig. 6. General scheme of the equilibria between enzyme, substrate, and inhibitor in relation to the effects of orphenadrine (*ORPH*), without preincubation, on pentoxyresorufin *O*-dependentylase (*PR*) activity in PB-induced microsomes. In this scheme the variable α is the factor by which K_s (substrate affinity constant) and K_i (inhibitor affinity constant) are altered in the presence of inhibitor and substrate, respectively.

activity in untreated microsomes. This suggests, therefore, that orphenadrine may selectively complex and inhibit P-450 PB-B rather than PB-D.

Orphenadrine was found to elicit significant induction of cytochrome P-450 and steroid 16β- and 6β-hydroxylase activities (probably mediated by the P-450s PB-B/D and PCN-E, respectively) and to increase P-450 PB-B immunoreactive protein content. In addition, treatment of hepatic microsomes isolated from orphenadrine-dosed rats with FCN resulted in a further elevation of total P-450 and androstenedione 16α- and 16β-hydoxylase activities over those activities in the absence of FCN. This finding is consistent with the dissociation of a complex generated *in vivo* between an orphenadrine metabolite and P-450 PB-B/D. Indeed, from the data presented in Tables 4 and 5 it can be estimated that the release of 0.3 nmol of P-450, following FCN treatment of microsomes from orphenadrine-treated rats, would result in the activation of 0.32 nmol/min/mg of protein) of androstenedione 16β-hydroxylase activity. Because the reactivation of androstenedione 16β-hydroxylase activity that was observed was 0.4 nmol/min/mg of protein and was in good agreement with the calculated value, further support is provided for the assertion that orphenadrine complexes selectively with P-450 PB-B *in vivo*. Additional evidence, obtained in experiments where the absorbance difference spectrum of orphenadrine-induced microsomes was scanned repetitively after FCN addition, was also consistent with formation of an *in vivo* MI complex. The trough at 456 nm in the difference spectrum is of a wavelength similar to that for other alkylamine metabolite-ferrous P-450 complexes. It is, therefore, likely that the nature of the orphenadrine complex is similar to that involving other alkylamine agents.

In view of the findings that androstenedione 16α- and 16β-hydroxylase activities were elevated by FCN treatment of orphenadrine-induced microsomes and that pentoxyresorufin *O*-dependentylation and androstenedione 16α- and 16β-hydroxylation were increased further after FCN treatment of PB-induced hepatic microsomes from rats dosed with orphenadrine, it would appear that P-450 PB-B/D is complexed *in vivo* by an orphenadrine metabolite. It is unlikely that complexation of the male-specific constitutive P-450 UT-A, which is active in steroid 16α-hydroxylation, occurs *in vivo*, because no complexation was observed in control microsomes [P-450 UT-A constitutes some 20–25% of total P-450 (19)]. Besides this point, *in vitro* inhibition experiments would argue against the involvement of P-450 UT-A because control microsomal steroid 16α-hydroxylase activity was not susceptible to inhibition by orphenadrine. The increase in androstenedione 16α-hydroxylation following FCN treatment, therefore, most likely results from the dissociation of an MI complex between orphenadrine and P-450 PB-B/D.

Orphenadrine administration was associated with 4.7- and 2.4-fold increases in androstenedione 16β- and 6β-hydroxylation, respectively. Because it now seems clear that the P-450 forms PB-B/D and PCN-E are the 16β- and 6β-hydroxylases, respectively (19), it would appear that P-450 PCN-E is present in the uncomplexed state following orphenadrine administration, whereas P-450 PB-B/D is complexed. There appears to be no evidence of complexation of either P-450 UT-A (the constitutive steroid 16α-hydroxylase) or P-450 UT-F (the 7α-hydroxylase). P-450 UT-F has also recently been shown to be resistant to SKF 525-A complexation (7). From these results it appears, therefore, that orphenadrine induces P-450 PB-B/D, and probably also P-450 PCN-E, but generates an MI complex with P-450 PB-B/D in a selective fashion.

Little information is available concerning the P-450 isozyme specificity of complex formation with nitrogen-containing compounds. Recent work in this laboratory (7) strongly suggests that SKF 525-A complexes with P-450s PB-B/D, PCN-E, and UT-A. Halpert (31) has shown that the macrolide antibiotic triacetyloleandomycin complexes specifically with P-450 PCNa. With each of these compounds as well as numerous other alkylamines, the nitroso derivative is thought to be responsible for MI complex formation (9–11). Thus, although the ultimate reactive species is the same in each case, the resulting complexation of P-450 is not uniform. This suggests, therefore, that nitroso formation may be catalyzed by multiple hepatic P-450 isozymes. Because it is unlikely that the highly reactive/unstable nitroso derivative can migrate from its site of formation, the selectivity of complexation is then determined by P-450 isozyme substrate specificity. A P-450 isozyme for which the nitroso precursor (probably the hydroxylamine) is a substrate, therefore, becomes susceptible to complexation. It follows then that the extent and rate of complexation of any one isozyme is dependent on the formation of its nitroso-precursor substrate, which, in turn, is influenced by the P-450 composition of the microsomal membrane. Thus, in the case of orphenadrine, at least four different reactions, which may be catalyzed by four different P-450s, are necessary for the formation of the nitroso precursor, the hydroxylamine (11). Induction or down-regulation of any of the P-450 isozymes involved in orphenad-

rine metabolism may, therefore, substantially influence complex formation. However, in the case of orphenadrine, it appears that the hydroxylamine is a selective substrate of P-450 PB-B/D.

Studies of this type, in which the P-450 isozymes involved in MI complex formation are identified, may ultimately lead to the prediction of such interactions, thus avoiding circumstances that may result in toxic insult. Cytochrome P-450 isozymes analogous to the major phenobarbital-inducible forms in rat (i.e., PB-B/D) have been identified in human liver (32). Thus, the selective complexation and/or inhibition of a human P-450 analogous to rat form P-450 PB-B/D may explain the observed increase in elimination half-life of orphenadrine, following a multiple-dose regimen (14).

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