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Coinduction of Multiple Hepatic Cytochrome P-450 Proteins and Their mRNAs in Rats Treated with Imidazole Antimycotic Agents

KEITH A. HOSTETLER, STEVEN A. WRIGHTON, DAVID T. MOLOWA, PAUL E. THOMAS, WAYNE LEVIN, and PHILIP S. GUZELIAN

Life Science Research, Hershey Foods Corporation, Hershey, Pennsylvania 17033 (K.A.H.), Department of Pharmacology and Toxicology, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 (S.A.W.), Department of Biochemistry and Molecular Pharmacology, Merck, Sharp, and Dohme Research Laboratories, Rahway, New Jersey 07065 (D.T.M.), Laboratory of Experimental Carcinogenesis and Metabolism, Roche Institute of Molecular Biology, Nutley, New Jersey 07110 (P.E.T., W.L.), and Division of Clinical Toxicology and Environmental Medicine, Department of Medicine, Medical College of Virginia, Richmond, Virginia 23298 (P.S.G.)

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SUMMARY

To characterize the molecular basis by which imidazole antimycotic drugs increase cytochrome P-450, we examined the effects of treating female rats with clotrimazole, miconazole, or ketoconazole on expression of the major inducible forms of hepatic cytochromes P-450 (P-450p, P-450b/e, P-450c/d, and P-450j). From measurements of the content of immunoreactive cytochromes P-450 in liver microsomes and of the amounts of liver RNA hybridizing to cloned P-450 cDNAs, we established that the glucocorticoid-responsive P-450p is the form predominantly induced by clotrimazole, miconazole, and ketoconazole, to as much as 382 times above control values. The phenobarbital-responsive cytochromes P-450b/e were also induced strongly by clotrimazole and miconazole, but not by ketoconazole. Aromatic hydrocarbon-inducible cytochromes P-450c/d were modestly elevated by each of these three antifungal drugs whereas ethanol-responsive P-450j was marginally induced by ketoconazole, but not by clotrimazole or miconazole. In some, but not all cases, treatment of rats with antifungal drugs resulted in accumulation of P-450 protein that significantly exceeded the increase in the corresponding P-450 mRNA. In conclusion, imidazole antifungal drugs differentially modulate the expression of at least four distinct gene subfamilies of rat hepatic cytochrome P-450 by separate mechanisms involving accumulation of P-450 mRNA and protein.

Oxidative biotransformation of lipophilic compounds is catalyzed by membrane-bound hemeproteins abundant in the endoplasmic reticulum of hepatocytes. The existence of multiple isozyme forms of these cytochromes P-450 partially accounts for the unusually broad substrate specificity exhibited by this enzyme system (1). Indeed, the cytochromes P-450 are now recognized as a superfamily of isozymes, which can be subdivided into distinct families according to common structural, functional, or regulatory characteristics. Many of these isozymes are inducible by hormones, drugs, and other xenobiotics (2). For example, barbiturates such as phenobarbital, polycyclic aromatic hydrocarbons such as β-napthoflavone, glucocorticoids and antiglucocorticoids such as pregnenolone-16α-carbonitrile, and ketones and alcohols such as ethanol are examples of four different classes of agents that selectively induce isozymes representing at least four different cytochrome P-450 subfamilies in rat liver (3).

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Recently, *N*-substituted imidazole compounds have been shown to increase the total amount of cytochrome P-450 in the liver (4–7). Several *N*-substituted imidazole derivatives are well known to be potential inhibitors of oxidative metabolism (8). These clinically useful antifungal agents interrupt synthesis of the major fungal sterol ergosterol at the cytochrome P-450-mediated step of lanosterol-14α-demethylation (9, 10). When one of these drugs, such as clotrimazole, miconazole, or ketoconazole is administered to rats, there is a rise in the capacity of liver microsomes to oxidize typical substrates of the cytochromes P-450, including erythromycin, ethylmorphine, aminopyrine, *N*,*N*-dimethylaniline, or *p*-nitroanisole (4–7). Analysis of the profile of induced drug-oxidizing activities suggests that clotrimazole may be a steroid-like inducer of cytochrome P-450 (5–7) and miconazole may possess both steroid and phenobarbital inducing characteristics (4, 7). However, it has been reported that ketoconazole induces a profile of microsomal activities distinct from those in phenobarbital-, steroid-, or methylcholanthrene-treated rats (4).

In the present study, we investigated the identity of the cytochrome P-450 isozymes regulated by these antimycotic drugs, by analyzing liver microsomes on immunoblots devel-

ABBREVIATIONS: TAO, triacetyloleandomycin; PBS, phosphate-buffered saline.

oped with specific antibodies directed against the major form(s) of cytochrome P-450 induced by glucocorticoids (P-450p), by phenobarbital (P-450b/e), by β-napthoflavone (P-450c/d), or by ethanol (P-450j). In addition, we have carried out northern blot analyses of liver RNA to determine the amounts of mRNA hybridizable to cloned cDNA probes to P-450p, P-450b/e, P-450c, P-450d, and P-450j. Our results reveal that at least four distinct subfamilies of rat liver cytochromes P-450 are induced by clotrimazole, miconazole, and ketoconazole. Moreover, there was a lack of proportionality between induction of immunoreactive P-450 protein, compared with corresponding increases in hybridizable P-450 mRNA, suggesting that these drugs selectively induce some cytochromes P-450 by effects on both mRNA and protein metabolism.

Experimental Procedures

Materials. TAO and ketoconazole were gifts from Pfizer Laboratories (Brooklyn, NY) and Janssen Pharmaceutica (Piscataway, NJ), respectively. Sodium phenobarbital was purchased from the Amend Drug and Chemical Co. (Irvington, NJ); clotrimazole, dexamethasone, β-napthoflavone, and miconazole nitrate were from Sigma Chemical Co. (St. Louis, MO). Melting point determinations were performed on each of the antifungal drugs to verify purity. Crisco brand cholesterol-free corn oil was purchased locally. Nitrocellulose was purchased from Bio-Rad (Richmond, CA); diaminobenzidine tetrahydrochloride from Pfaltz & Bauer (Stamford, CT); goat peroxidase antiperoxidase from Miles Scientific (Elkhart, IN); and peroxidase anti-mouse IgG, peroxidase anti-rabbit IgG, and rabbit anti-goat IgG from ICN Immuno-Biologicals (Lisle, IL). All other reagents were of the finest grade commercially available.

Animals and treatments. Female Sprague-Dawley rats (175–225 g; Flow Laboratories, Dublin, VA) were housed in pairs in wire-bottom cages and given unlimited access to food and water. Each compound was suspended in corn oil (except sodium phenobarbital, which was dissolved in 0.9% sodium chloride) and was administered by oral gavage (except phenobarbital and β-napthoflavone, which were given by intraperitoneal injection). Miconazole (150 mg/kg), ketoconazole (150 mg/kg), clotrimazole (100 or 150 mg/kg), phenobarbital (80 m/kg) dexamethasone (300 mg/kg), and β-napthoflavone (80 mg/kg) were administered daily for 3 days. TAO (480 mg/kg) was given daily for 5 days. Vehicle-treated rats were given a daily dose of corn oil, 10 ml/kg, orally for 3 days. Ethanol was provided at 6.4% in a Lieber and DeCarli liquid diet (11) for 2 days. Corresponding controls were provided an isocalorically equivalent diet without ethanol.

Animals were fasted overnight and then killed by decapitation 24 hr after the final treatment, unless otherwise noted. Each liver was perfused with iced PBS, pH 7.4, and then was excised and homogenized with a motor-driven Teflon pestle in a buffer containing 100 mM Tris, 100 mM KCl, 1 mM EDTA, and 20 μM butylhydroxytoluene, pH 7.4. Microsomes were isolated by differential centrifugation as previously described (12) and stored immediately at −70°. Total CO-binding cytochrome P-450 content of each microsomal sample was determined as the dithionite-reduced CO difference spectrum (13) under experimental conditions identical to those described in the indicated published procedure (14). The sample was saturated with CO for 60 sec and sequential difference spectra were recorded until the absorbance at 450 nm reached a maximum. Microsomes from TAO-treated rats were decomplexed with $K_3Fe(CN)_6$, as previously described (15). Microsomal protein concentrations were determined colorimetrically (16).

Isolation of purified proteins and antibodies. Rat liver cytochromes P-450p (17), P-450b (18), and P-450j (19) were purified according to the indicated published procedures. A monoclonal antibody directed against purified P-450p was prepared and characterized as described (20). The polyclonal antibody directed against P-450b was raised in a goat as described (21) and then processed by sequential immunoabsorptions, first against liver microsomes from β-napthoflavone-treated rats and then against microsomes from untreated male rats. A form-specific polyclonal antibody raised against the major aromatic hydrocarbon-inducible rat liver cytochromes (P-450c/d) was prepared and characterized as previously described (12). Form-specific anti-P-450j antibody was raised in rabbits and has been well characterized (22).

Immunoblot analyses. Liver microsomes were resolved in polyacrylamide slab gels and blotted onto nitrocellulose filters. After an overnight incubation in blocking solution consisting of PBS with 3% bovine serum albumin and 10% calf serum, development of each blot was initiated with a 60-min exposure to the appropriate antibody. Exposure of blots to anti-P-450p or anti-P-450j antibody was followed by a 30-min incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG, respectively. Immunoblots developed with either anti-P-450b or anti-P-450c/d required sequential 30-min incubations with rabbit anti-goat IgG and peroxidase goat anti-peroxidase. Nitrocellulose filters were washed extensively with PBS between successive antibody incubations. Immunoreactive proteins were visualized with *N,N*-diaminobenzidine in 0.006% H_2O_2. For quantitative immunoblot analyses, electrophoretic transfer was performed under previously established conditions, which insure complete transfer of proteins in the 40–60 kDa range (20, 23). Next, the filters were incubated with the appropriate antibody and then were stained. The integrated absorbance of each stained protein band was determined by scanning densitometry. Before final quantitation experiments, the linear range of absorbance per microgram of protein analyzed was established for each antibody and each treatment.

Isolation and analyses of RNA. The P-450p (15), P-450b/e (24), P-450c and P-450d (25), and P-450j (26) cDNA probes were isolated and characterized as in the indicated references. Total liver RNA from control or treated rats was isolated using the guanidine isothiocyanate method (21, 27). Northern blot analyses were carried out as described in a previous report (15). Briefly, aliquots of RNA from each animal were resolved by electrophoresis in agarose gels and then transferred to nitrocellulose filters. Each filter was baked under vacuum, prehybridized, and then incubated in a hybridization solution containing ^{32}P-labeled cDNA probes, as described previously (28). Hybridization of the labeled cDNA probes to filter-bound RNA was visualized by autoradiography. Quantitation of hybridizable P-450 mRNA was accomplished using a Schliecher and Schuell slot blot apparatus under conditions recommended by the manufacturer. RNA samples were diluted and applied directly to nitrocellulose filters, which were further processed as described above for Northern blot filters. For each treatment group and each cDNA probe, an appropriate range of RNA was analyzed that permitted calculation of a linear slope value (absorbance per microgram of RNA analyzed) after hybridization, autoradiography, and scanning densitometry. Slope values were determined for each treated rat and were compared with slope values for control female rats analyzed on the same filter.

Statistical methods. Two-tailed Student *t* tests were performed on unpaired sample means and the significance was determined at the level of $p \leq 0.05$.

Results

Effects of treatments on total CO-binding cytochrome P-450. Clotrimazole, miconazole, and ketoconazole have each been identified as inducers of total spectrally determined cytochrome P-450 (4–7), although the maximum tolerated dose of each of these compounds has not been reported. In preliminary studies of these agents, we have found that clotrimazole, when administered at 150 mg/kg daily for 3 days, produced a remarkable induction of total P-450, about 5-fold over control values (Fig. 1). This induction was even greater than that produced by TAO (Fig. 1), a macrolide antibiotic inducer of P-450p that, until now, was the most efficacious inducer of rat

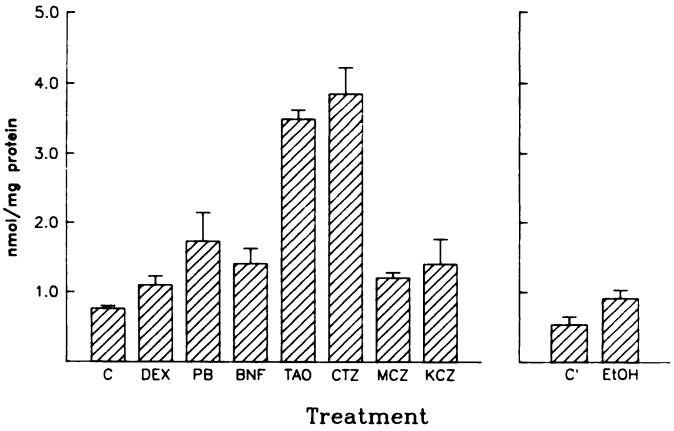

Fig. 1. Total CO-binding P-450 in liver microsomes. Rats were treated with inducers (*DEX*, dexamethasone; *PB*, phenobarbital; *BNF*, β-napthoflavone; *CTZ*, clotrimazole, *MCZ*, miconazole; *KCZ*, ketoconazole; *EtOH*, ethanol, or vehicle (*C*, corn oil control, *left*; *C'*, Lieber-DeCarli control diet without ethanol, *right*) as described in Experimental Procedures. Liver microsomes were isolated by differential centrifugation and total spectral P-450 was determined by carbonyl-reduced difference spectroscopy. Sequential spectra were recorded until the absorbance at 450 nm reached a maximum. Microsomes from TAO-treated rats were decomplexed by adding $K_3Fe(CN)_6$ to a final concentration of 20 μM, as previously described (16). Shown are the mean ± the standard deviation of values from five individual rats.

liver cytochrome P-450 ever reported (17, 20). Treatment of rats with the maximum tolerated dose (150 mg/kg) of ketoconazole or miconazole increased cytochrome P-450 over control values by 1.6- and 1.8 fold, respectively, similar to increases following treatment with prototype inducers such as dexamethasone (2.3-fold), phenobarbital (1.9-fold), or ethanol (1.7-fold) (Fig. 1). We noticed that only 2 to 3 min was required to achieve the peak absorbance at 450 nm after CO saturation of the sample cuvette, except for samples from clotrimazole-treated rats, which required 16 to 24 min. Others have also found a slowly developing difference spectrum, indicative of competition between CO and a compound tightly bound to heme (7, 29). We treated additional rats with a lower dose of clotrimazole (100 mg/kg daily for 3 days) and waited 42 hr after the final clotrimazole treatment to isolate liver microsomes, instead of the routine 24 hr. The extent of induction of total CO-binding cytochrome P-450 in these microsomes was unchanged with the use of this protocol (data not shown). Nevertheless, despite the extended delay between the final clotrimazole treatment and microsome isolation, the putative residual competitor was still present, as evidenced by a requirement of 6 to 12 min before the CO difference spectrum reached a maximum.

Identification and quantitation of immunoreactive cytochromes P-450. Previous investigations of clotrimazole, miconazole, and ketoconazole as inducers of rat liver cytochrome P-450 have principally relied on microsomal catalytic activity measurements (4–7). Following preliminary experiments that verified published findings (data not shown), we chose to evaluate cytochrome P-450 induction more directly by measuring the amounts of cytochrome P-450 recognized by specific antibody probes in liver microsomes from control and induced rats. Representative immunoblots developed with antibodies directed against P-450p, P-450b, P-450c/d, and P-450j are shown in Fig. 2 and the results of quantitative immunoblot analyses are summarized in Table 1. These studies revealed that total immunoreactive P-450p in microsomes was markedly increased (more than 380-fold over control values) by clotrimazole (Table 1). Clotrimazole induced immunoreactive P-450p in excess of that induced by TAO (221-fold; Table 1). At the doses that maximally elevated total CO-binding cytochrome P-450, miconazole and ketoconazole were less effective inducers of immunoreactive P-450p (52- and 54-fold, respectively) and were intermediate between dexamethasone (124-fold) and phenobarbital (26-fold; Table 1).

The same liver microsomal samples were quantitatively analyzed on immunoblots developed with anti-P-450b, anti-P-450c/d, or anti-P-450j antibodies (Table 1). Immunoreactive P-450b in vehicle-treated female rats was below the limit of quantitative detection for the antibody employed. Thus, calculation of the "fold increases" over control values of anti-P-450b-reactive protein following treatments was not possible. Nevertheless, treatment of rats with either clotrimazole or miconazole produced a striking increase in immunoreactive P-450b protein (Fig. 2; Table 1). Indeed, at the doses we tested, clotrimazole and miconazole each increased immunoreactive P-450b protein to levels in the same range as did phenobarbital (Table 1). In contrast, ketoconazole did not significantly elevate P-450b/e protein above the lowest detectable amount (Table 1).

N-Substituted imidazole treatments also stimulated the expression of the aromatic hydrocarbon-responsive cytochromes P-450c/d (Fig. 2; Table 1). Treatment of rats with the prototype P-450c/d inducer β-napthoflavone elevated total immunoreactive P-450c/d about 20 times over control values. In contrast, only modest elevations in microsomal content of anti-P-450c/d-reactive protein (2- to 4-fold over control values) occurred following treatment with clotrimazole, miconazole, or ketoconazole (Table 1). The major immunoreactive band in microsomes from antimycotic-treated rats comigrated with the fastest migrating β-napthoflavone-inducible protein, presumably cytochrome P-450d (Fig. 2)(30).

Among the major inducible rat liver cytochromes P-450, the ethanol-responsive P-450j was affected the least by imidazole antimycotics at the doses administered in this study. Levels of immunodetectable P-450j protein were increased by the prototype inducer ethanol and by ketoconazole but were not increased by either clotrimazole or miconazole (Table 1). The increases that were observed in P-450j expression were marginal ones (about 2-fold) when comparing with the striking elevations in immunoreactive P-450p and P-450b/e resulting from treatment with imidazole antimycotic agents.

Analyses of cytochrome P-450 mRNA. Representative Northern blots of liver RNA isolated from control and treated rats revealed differential hybridization signals, depending upon which of the P-450 cDNA probes was employed (Fig. 3). In each set of experiments, the ^{32}P-labeled cDNA probe utilized was found to hybridize with a single major mRNA species, thus permitting quantitative slot blot analyses of total hybridizable mRNA. The results, which are expressed as the ratio of hybridization signals in treated versus control rats, are summarized in Table 2. The data from Tables 1 and 2 are depicted together in Fig. 4, as ratios (treated/control) of P-450p, P-450d, and P-450j protein and mRNA. (P-450b/e was excluded because the protein content of these isozymes in control rats is below the limit of detection.) It was apparent that clotrimazole treatment resulted in an increase in hybridizable P-450p mRNA that was much less than the corresponding increase in immunoreactive

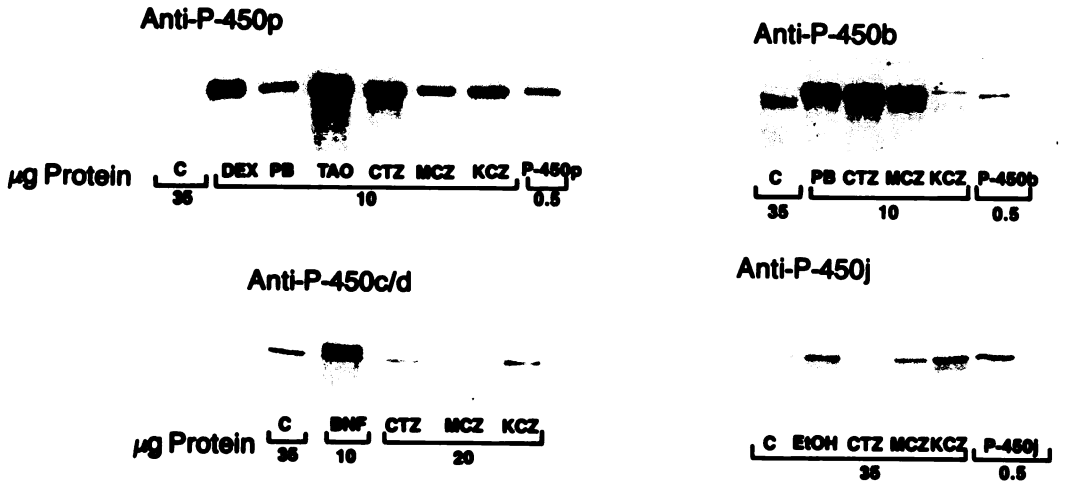

Fig. 2. Immunoblot analyses of liver microsomes. Microsomal samples from control or induced female rats containing the indicated protein quantities were electrophoresed, blotted, and then developed with the indicated antibodies as described in Experimental Procedures. Also included as mobility standards are purified P-450p, P-450b, and P-450j.

TABLE 1

Quantitation of immunoreactive cytochromes P-450 in liver microsomes from rats treated with inducers

Quantitative immunoblot analyses of microsomes isolated from control or treated rats were performed as described under Experimental Procedures. The results are expressed as total immunoreactive protein, in densitometric units per microgram analyzed, along with the ratio of treated to control values. Shown are the mean ± the standard deviation of values from five individual animals for each control or treatment group. Abbrevations used are outlined in Fig. 1.

Treatment	Anti-P-450p		Anti-P-450b		Anti-P-450c/d		Anti-P-450j	
	U/μg[a]	Tx/ctrl[b]	U/μg	Tx/ctrl	U/μg	Tx/ctrl	U/μg	Tx/ctrl
Vehicle	0.008 ± 0.001		<0.006		0.059 ± 0.007		0.045 ± 0.016	
CTZ	3.054 ± 0.432	382	0.514 ± 0.190	NA[c]	0.239 ± 0.100	4.1	0.031 ± 0.004	0.69
MCZ	0.414 ± 0.116	52	0.613 ± 0.125		0.124 ± 0.081	2.1	0.039 ± 0.013	0.87
KCZ	0.429 ± 0.150	54	0.007 ± 0.002		0.247 ± 0.113	4.2	0.095 ± 0.011[d]	2.10
DEX	0.988 ± 0.318	124						
TAO	1.764 ± 0.447	221						
PB	0.211 ± 0.049	26	0.476 ± 0.132					
BNF					1.163 ± 0.402	19.7		
EtOH							0.115 ± 0.011[d]	2.56

[a] Densitometric units per microgram of protein.
[b] Treated/control.
[c] NA, not applicable (control below lowest detectable amount).
[d] $p < 0.05$, compared with vehicle-treated control.

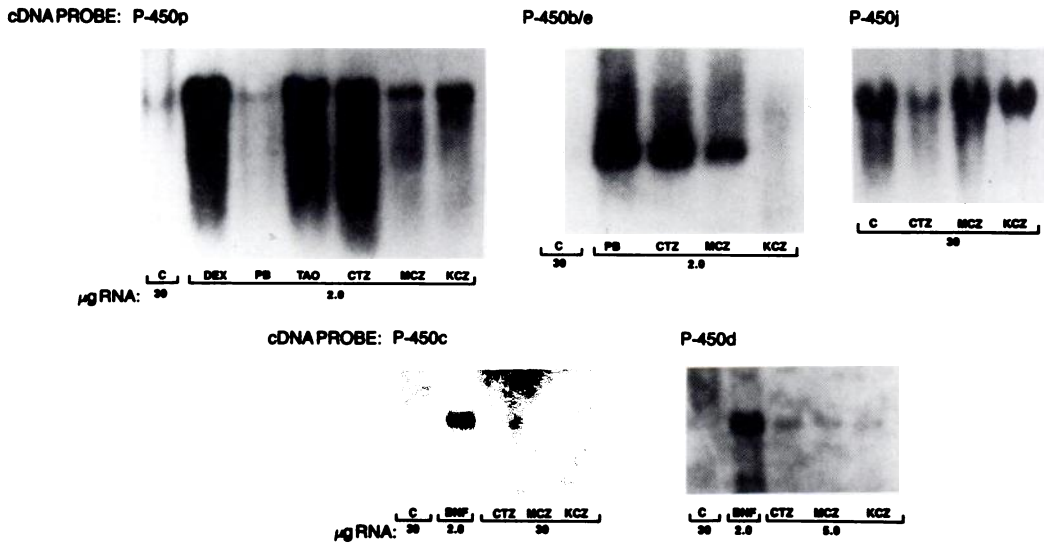

Fig. 3. Northern blot analyses of hybridizable P-450 mRNA. Total liver RNA isolated from rats treated as outlined in Fig. 1 was subjected to electrophoresis in agarose gels, transferred to nitrocellulose filters, and then hybridized with the indicated ^{32}P-labeled cDNA probe as described in Experimental Procedures. Hybridizable P-450 mRNA was visualized by autoradiography.

P-450p protein (Fig. 4). Similarly, TAO produced a disproportionate increase in immunoreactive P-450p protein, as compared with hybridizable P-450p mRNA (Fig. 4). In contrast, miconazole, ketoconazole, dexamethasone, and phenobarbital each produced similar increases over control values of P-450p protein and mRNA (Fig. 4). Hybridizable P-450b/e mRNA was increased by clotrimazole and by miconazole, but neither drug caused as great an increase as did phenobarbital (Table 2).

Modest increases in hybridizable P-450d mRNA, (2- to 4-fold) were observed in samples from rats treated with either clotrimazole, miconazole, or ketoconazole (Table 2). These changes closely parallelled increases in immunoreactive protein, which indicated that the major antimycotic-induced protein band is P-450d and not P-450c. Indeed, hybridizable P-450c mRNA from these same treated animals was found to be below control levels (Table 2). None of the agents we tested

TABLE 2

Quantitation of hybridizable P-450 mRNA

Samples of total liver RNA identical to those analyzed in Fig. 3 were subjected to slot blot analyses as described in Experimental Procedures. RNA samples from each rat (0.05 to 30 μg) were applied to nitrocellulose filters, hybridized with the indicated ^{32}P-labeled cDNA probe, visualized by autoradiography, and quantitated by scanning densitometry. The results are expressed as the ratio of treated to control values and represent the mean of values from three to five individual animals. Abbreviations used are outlined in Fig. 1.

Treatment	P-450p	P-450b/e	P-450c	P-450d	P-450j
			treated/control		
CTZ	92.9	23.9	0.61	2.50	0.70
MCZ	31.9	18.8	0.36	1.91	1.00
KCZ	58.2	0.9	0.35	2.76	0.70
DEX	140.8				
TAO	44.8				
PB	28.1	36.0			
BNF			13.4	28.3	

increased hybridizable P-450j mRNA, including ketoconazole, the only imidazole that induced immunoreactive P-450j protein (Fig. 4).

Discussion

The results demonstrate the *N*-substituted imidazole drugs clotrimazole, miconazole, and ketoconazole represent a new class of inducers of the glucocorticoid-responsive family (P450III) of hepatic cytochromes P-450 in the rat. We found remarkable accumulation of immunoreactive P-450p protein, along with increased amounts of hybridizable P-450p mRNA in livers of rats treated with clotrimazole, miconazole, or ketoconazole. These drugs also increase microsomal *N*-demethylation of erythromycin (5–7), an activity linked to P-450p (17). Indeed, clotrimazole now supplants TAO as the most effective known inducer of total CO-binding hemeprotein and of P-450p (17, 20).

Several years ago, we investigated the mechanism underlying the dramatic induction of cytochrome P-450p by the macrolide antibiotic TAO and found that both in hepatocyte cultures and in living rats, this drug selectively prolongs the half-life of P-450p (31). Thus, TAO treatment results in a marked accumulation of P-450p protein while P-450 mRNA is increased only slightly (17, 31). In the present studies, the administration of clotrimazole to rats also produced a disproportionately large increase in P-450p protein relative to the rise in hybridizable P-450p mRNA (Fig. 4). Thus, it seems likely that clotrimazole, like TAO, interrupts the degradation of P-450p. TAO is known to be converted by P-450p to a metabolite that then binds tightly to P-450p and inhibits its further catalytic activity (17, 31). Clotrimazole does not appear to form this same type of complex with cytochrome P-450, detectable by its characteristic spectral features (6). In this regard, clotrimazole resembles other types of P-450p inducers such as chlordane or *trans*-nonachlor, which increase the accumulation of P-450p protein to a greater extent than they increase its rate of *de novo* synthesis and yet do not form a stable metabolite complex with P-450p (28). Miconazole and ketoconazole, in contrast to clotrimazole, each increased P-450p protein and mRNA to similar extents, as did dexamethasone and phenobarbital. These findings suggest that imidazole antifungal drugs may be useful probes for further study of the control of P-450p turnover.

When we compared the magnitude of induction of P-450p mRNA and protein by dexamethasone (Tables 1 and 2) with that reported earlier by Simmons and co-workers (32), a notable discrepancy became apparent. These investigators found that a single dexamethasone injection increased the amount of P-450p-related mRNA (measured as cytoplasmic poly(A)$^+$ mRNA hybridizable to a P-450PCN cDNA probe) a maximum of 18-fold over control values, whereas chronic dexamethasone administration (80 mg/kg daily for 4 days) elevated immunodetectable P-450p-related protein only 4-fold. In contrast, we found that dexamethasone induced P-450p mRNA and protein 141- and 124-fold, respectively (Tables 1 and 2). It is possible that differences in gender of rats treated, doses and routes of administration of the steroid, methods of immunoquantitation, and specificities of antibodies utilized may account for the observed discrepancies.

The cytochrome P-450-inducing properties of the antimycotic drugs we investigated are not limited to the P-450p family. Our immunochemical and cDNA hybridization studies revealed that clotrimazole and miconazole, but not ketoconazole, are also effective inducers of the phenobarbital-inducible cytochromes P-450b/e. Indeed, our findings on the effects of clotrimazole, miconazole, and ketoconazole on steroid- and pheno-

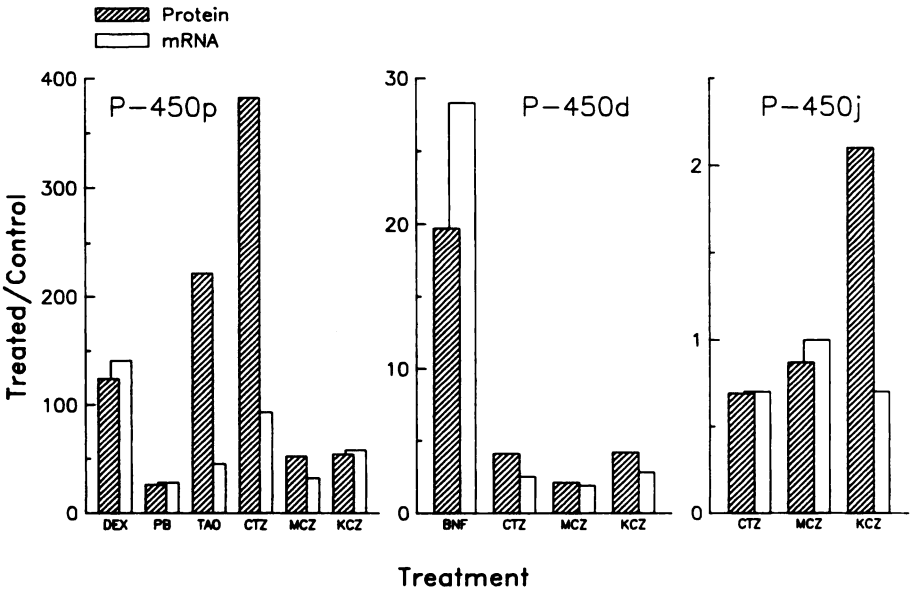

Fig. 4. Effect of treatments on P-450 protein and mRNA. Ratio of treated to control values from immunoquantitation and RNA hybridization studies with P-450p (*left*), P-450d (*center*), and P-450j (*right*) antibody and cDNA probes. Quantitations were carried out as described in Experimental Procedures. The data shown are derived from Tables 1 and 2.

barbital-inducible cytochromes P-450 are in close agreement with those reported recently by Rodrigues and co-workers (33). Thus, these agents can be grouped along with others such as chlordane, *trans*-nonachlor, halogenated biphenyls, and phenobarbital as inducers shared by the steroid- and phenobarbital-responsive cytochrome P-450 families. The findings we report here add to the mounting evidence that, in rat liver, these two distinct cytochrome P-450 gene families exhibit overlapping regulatory characteristics.

The expression of yet another rat liver P-450 family of isozymes, those induced by aromatic hydrocarbons, was also stimulated by *N*-substituted imidazoles, albeit to a lesser extent than were members of the steroid- and phenobarbital-responsive families. Results from both protein and mRNA analyses suggested that the effects on this family were somewhat selective for P-450d. In this regard, clotrimazole, miconazole, and ketoconazole resemble isosafrole, another agent that preferentially induces P-450d (34). Finally, P-450j, an isozyme representing a fourth separate rat liver cytochrome P-450 subfamily, was induced by ketoconazole but *not* by either clotrimazole or miconazole. Induction of P-450j by ketoconazole, as evidenced by a modest increase in immunodetectable protein, occurred in the absence of any change in the level of hybridizable P-450j mRNA (Fig. 4). Thus, ketoconazole, like other inducers of P-450j, such as pyrazole, acetone, or 4-methylpyrazole, appears to affect the expression of this isozyme by posttranslational mechanisms (26, 35). Moreover, it can be reasonably concluded that, as a class of P-450 inducers in the rat, the *N*-substituted imidazoles regulate the expression of at least four different subfamilies of cytochrome P-450 isozymes by multiple mechanisms, one of which apparently involves altered rates of protein turnover.

In summary, our present findings add to the growing body of evidence that *N*-substituted imidazoles represent a unique and important class of agents that have multiple effects on the expression of hepatic cytochromes P-450 in the rat. These drugs will undoubtedly serve as useful tools in the ongoing investigation of the molecular mechanisms involved in the regulation of hepatic cytochromes P-450. Obviously, the doses of clotrimazole, miconazole, and ketoconazole administered to rats in this study far exceed clinically relevant doses of these compounds. Nevertheless, it is clear that the potential exists for significant alterations in human metabolism during antimycotic therapy. Indeed, there have been numerous reports of ketoconazole-associated hepatic injury (36). Recently, in addition to its use as an antifungal agent, ketoconazole has been utilized as an inhibitor of steroid synthesis (37), further emphasizing the importance of identifying the mechanisms by which ketoconazole and structurally similar compounds regulate the expression of cytochromes P-450. Because clotrimazole and miconazole are primarily used as topical antimycotics and systemic absorption is limited, less has been reported about their effects on human metabolism. In conclusion, this study indicates that newly developed orally administered *N*-substituted antimycotics should be carefully examined as possible modulators of oxidative metabolism in humans.

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Send reprint requests to: Philip S. Guzelian, Division of Clinical Toxicology and Environmental Medicine, Department of Medicine, Medical College of Virginia, Box 267, MCV Station, Richmond, Virginia 23298.