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GLUCOCORTICOID-RESPONSIVE HEPATIC CYTOCHROMES P-450 IN THE RAT:
MULTIPLICITY AND DIFFERENTIAL REGULATION

A thesis submitted in partial fulfillment of the requirements for the
degree of Doctor of Philosophy at Virginia Commonwealth University

BY

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DEDICATION

To Kathy, for her constant support, understanding,
inspiration, and love.

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ABSTRACT

A thesis submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Virginia Commonwealth University.

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The multiplicity of glucocorticoid-responsive hepatic cytochromes P-450 in the rat was investigated by developing a series of monoclonal antibody probes directed against purified steroid-inducible isozymes from the rat (P-450p) and man (HLp). Two antibodies, termed 1G8 and 13-7-10, showed unique specificity for purified cytochromes P-450 upon immunoblot analyses. These antibodies were further characterized in terms of their reactivity toward proteins in liver microsomes from untreated and xenobiotic-treated rats. Three P-450p-related proteins were identified using 1G8 and 13-7-10 to monitor the expression of P-450p family subtypes. In untreated rats, members of this family exhibited sex-specific expression. Treatment with various chemicals resulted in differential induction of the 1G8- and 13-7-10-reactive proteins, demonstrating distinct regulatory features of these immunochemically-related proteins. While searching for novel inducers of the P-450p family, the imidazole antifungal drug clotrimazole was

found to significantly increase total cytochrome P-450. Detailed studies were undertaken to investigate the mechanism by which clotrimazole and other antifungal drugs induce hepatic cytochrome P-450. The effect of treatment of rats with the antifungal drugs clotrimazole, miconazole, or ketoconazole on the expression of three distinct P-450 gene families (glucocorticoid-responsive P-450p, phenobarbital-responsive P-450b/e, and ethanol-responsive P-450j) was determined by measuring: 1) microsomal enzymatic activities with marker substrates, 2) the microsomal content of immunoreactive cytochromes P-450 with specific antibodies, and 3) the amounts of liver RNA hybridizing to cloned P-450 cDNAs. These studies establish that P-450p is the predominant P-450 isozyme induced by clotrimazole, miconazole, and ketoconazole. Two of these drugs, clotrimazole and miconazole also induced P-450b/e, whereas ketoconazole induced P-450j. Differential increases in specific P-450 mRNAs and proteins resulted from treatment of rats with these imidazole antifungal drugs, suggesting that multiple cellular events are involved their mechanism of P-450 induction. In conclusion, expression of the P-450p family of cytochromes P-450 in rat liver is under complex regulatory control and is subject to modulation by clinically useful drugs.

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INTRODUCTION

Metabolism of foreign chemicals including drugs and other xenobiotics, along with important endogenous compounds, is dependent on catalysis by heme proteins abundant in the endoplasmic reticulum of hepatocytes collectively termed the cytochromes P-450 (Guengerich, 1979; Lu & West, 1980; Conney, 1982; and Coon & Koop, 1983). The unusually broad substrate specificity exhibited by these enzymes is partially accounted for by the existence of multiple forms, or isozymes, each encoded by a separate gene (Adesnick & Atchison, 1985). Another important feature which imparts versatility to this enzyme system is that some, but not all isozymes of cytochrome P-450 are inducible (Guengerich *et al.*, 1982). Thus, in response to a given inducer, the concentration of one or more isozymes increases and then returns to untreated levels upon clearance of the inducer. Indeed, the inducibility of xenobiotic metabolism was the basis for early classification schemes of both cytochrome P-450 forms and chemicals identified as P-450 inducers. For example, isozymes of cytochrome P-450 were initially believed to fall into one of three classes, a "control" or untreated form, a form inducible by barbiturates such as phenobarbital, or a form inducible by aromatic hydrocarbons such as 3-methylcholanthrene (Ryan, *et al.*, 1979).

As methods for isolating and purifying isozymes of cytochrome P-450 progressed, it became clear that rat liver contained more than three distinct forms of cytochrome P-450. Nevertheless, it remained customary to classify newly identified inducing chemicals as being

either "phenobarbital-like" or "3-methylcholanthrene-like." A third prototype inducer, the catatoxic steroid pregnenolone-16 α -carbonitrile or PCN, was subsequently identified and shown to induce a form of P-450 distinct from those induced by either phenobarbital or 3-methylcholanthrene (Elshourbagy & Guzelian, 1980). The major form of rat liver cytochrome P-450 induced by PCN, by definition termed P-450p, has since been the subject of extensive investigation and found to possess a number of unique features among P-450 isozymes. Studies of both cultured hepatocytes and the living rat have revealed that expression of P-450p is stimulated not only by PCN, but also by glucocorticoids, barbiturates, organochlorine pesticides, halogenated biphenyls, and macrolide antibiotics (Heuman et al., 1982; Schuetz et al., 1984; Schuetz & Guzelian 1984; Schuetz et al., 1986; Wrighton et al. 1985a). Moreover, functionally and structurally related cytochromes, or homologues, have been identified in all species examined to date including man (Wrighton et al., 1985b; Watkins et al. 1985).

A growing body of evidence has accumulated recently suggesting that in rats, P-450p is only one member of a family of closely related isozymes. Indeed, it is clear that the cytochromes P-450 are encoded by a superfamily of genes that can be divided into distinct families based on genetic, regulatory, and structural similarities (Whitlock, 1986; Nebert et al., 1987). In rat liver, one such family consists of 8 or more genes, several of which encode proteins that are inducible by phenobarbital, including cytochromes P-450b and P-450e (Kumar & Adesnick, 1983). Likewise, 3-methylcholanthrene-inducible cytochromes P-450c and P-450d, and ethanol-inducible cytochrome P-450j are representatives of other distinct isozyme families (Ryan et al., 1979;

Nebert et al., 1987). Evidence for the existence of a "PCN family" of cytochromes P-450 includes the observation that liver microsomes isolated from dexamethasone-treated rabbits contain two immunoreactive protein bands recognized by polyclonal antibodies raised against either rat P-450p or the rabbit homologue P-450LM3c (Wrighton et al. 1985b). In the same report, Northern blot analyses of liver RNA isolated from PCN-treated rats revealed at least two mRNA species hybridizing to a P-450p cDNA. Southern blot analyses of either the rat or human genome with the P-450p cDNA probe verified the existence of sufficient hybridizable DNA to encode four or more P-450p-related genes (Watkins et al., 1986). Finally, Gonzalez and coworkers isolated and characterized two nearly identical but distinct cDNAs from rats treated with PCN (Gonzalez et al. 1985). The deduced amino acid sequence of one of these clones is identical with the N-terminal sequence of P-450p. Nevertheless, no single laboratory has succeeded in isolating and purifying more than one steroid-inducible cytochrome P-450 from rat liver.

Based on these observations, it was hypothesized that multiple P-450p-related isozymes are expressed in rat liver. Chapter 1 of this thesis, entitled "Immunochemical Evidence for Multiple Steroid-Inducible Hepatic Cytochromes P-450 in the Rat," describes the characterization of two highly specific monoclonal antibody probes which react with two subtypes of structurally similar steroid-inducible cytochromes P-450. The data presented demonstrates that, although these proteins share common structural features, they are under complex and discoordinate regulatory control. Expression of the P-450p family of proteins is shown to be dependent not only upon recent exposure to a

variety of inducing chemicals, but also upon gender.

Studies undertaken to isolate and purify from rat liver one or more proteins distinct from but related to P-450p verified the extensive structural and physical similarities among members of this isozyme family. Indeed, each purification study has resulted in the co-purification of proteins from both subtypes of P-450p-like proteins. Nevertheless, a new and unique class of chemical inducers of hepatic cytochrome P-450 was identified in the course of screening novel inducing compounds. In Chapter 2, "Co-induction of Multiple Hepatic Cytochrome P-450 Proteins and mRNAs in Rats Treated with Imidazole Antimycotic Agents," three commonly used antifungal drugs are characterized in terms of their differential effects on the expression of three different families of hepatic cytochrome P-450. Investigation of the possible mechanism of induction of this class of compounds suggests that effects on both RNA and protein metabolism are involved.

CHAPTER ONE

Immunochemical Evidence for Multiple Steroid-Inducible
Hepatic Cytochromes P-450 in the Rat.

ABSTRACT

It has been established that there are glucocorticoid-inducible hepatic cytochromes P-450 in the rat (P-450p), the rabbit (LM3c), and man (HLp) which share extensive structural, functional, and regulatory features, and that in rats, P-450p may be a member of a family of closely related cytochromes. Immunochemical probes to P-450p were prepared and a unique monoclonal antibody, 1G8, was identified which recognizes purified P-450p, but neither purified LM3c nor HLp upon immunoblot analyses. Similar analyses of liver microsomes from untreated male rats revealed two 1G8-reactive proteins, whereas liver microsomes from untreated females contained none. Another monoclonal antibody, 13-7-10, reacted specifically with LM3c and HLp, but not with P-450p. A single 13-7-10-reactive microsomal protein was detected in untreated male and female rats, the latter protein exhibiting a greater apparent molecular weight. 1G8-reactive proteins were induced to the greatest extent by triacetyloleandomycin, followed by dexamethasone, chlordane, pregnenolone-16 α -carbonitrile, and 2,4,2',4'-tetrachlorobiphenyl. In contrast, 13-7-10-reactive proteins were most strongly induced by dexamethasone, only moderately by triacetyloleandomycin and pregnenolone-16 α -carbonitrile, weakly by chlordane and not at all by 2,4,2',4'-tetrachlorobiphenyl. In conclusion, the P-450p family in rat liver consists of three or more proteins that are structurally related and yet appear to be under distinct regulatory control.

INTRODUCTION

The liver cytochromes P-450 are a superfamily of hemoproteins located in the endoplasmic reticulum of the hepatocyte that play a critical role in the biotransformation of a diverse array of endogenous substances, such as steroid hormones, as well as xenobiotics, such as drugs and carcinogens (Lu & West, 1979; Adesnik & Atchison, 1985). Families of cytochrome P-450 proteins have been identified based on their similarities in biochemical, immunochemical, and genetic characteristics, including their responses to prototypic inducers. For example, P-450b and P-450e are immunochemically and structurally similar hemoproteins that are coinduced in the liver of rats treated with phenobarbital or phenobarbital-like inducers (Kumar et al., 1983; Adesnik & Atchison, 1985). Likewise, P-450c and P-450d are products of homologous genes whose expression is stimulated by administration of 3-methylcholanthrene and other polycyclic aromatic hydrocarbons (Adesnik & Atchison, 1985; Guengerich et al., 1982).

Several years ago, this laboratory identified a unique form of cytochrome P-450 (Elshourbagy & Guzelian, 1980), now termed P-450p, that is inducible by the synthetic steroid, pregnenolone-16 α -carbonitrile (PCN) and by steroids of the glucocorticoid class such as dexamethasone (Heuman et al., 1982; Schuetz et al., 1984). Cytochrome P-450p is structurally and functionally distinct from the "phenobarbital-inducible" and "methylcholanthrene-inducible" forms of cytochrome P-450, and yet P-450p is inducible by phenobarbital and "phenobarbital-like" inducers (Guengerich et al., 1982; Heuman et al.,

1982; Schuetz et al., 1986). Macrolide antibiotics represent a third group of compounds that induce P-450p (Wrighton et al., 1985a). Cytochromes immunochemically and functionally related to P-450p have been found in all species thus far examined (Wrighton et al., 1985b) including P-450 LM3c in the rabbit and P-450 HLp in man (Watkins et al., 1985). Cytochrome P-450p may be involved in important biotransformation reactions because it is conserved among mammalian species and is regulated by an endogenous inducer (corticosterone) (Schuetz & Guzelian, 1984).

It is now evident that P-450p is a representative of a multi-gene cytochrome P-450 family. Cloned cDNA's to P-450p hybridize to at least two liver RNA species in PCN-treated rats (Wrighton et al., 1985b) and to a sufficient amount of the rat genome to account for as many as five P-450p-related genes (Molowa et al., 1986; Hardwick et al., 1983). Until recently, the liver cytochromes isolated by others from PCN-treated rats [P-450PB/PCN-E (Guengerich et al., 1982) and P-4502a (Waxman, 1986)] appeared to be the same as P-450p. Gonzalez and co-workers identified and isolated a PCN-inducible protein and corresponding cDNA (P-450PCN1) (Gonzalez et al., 1985) apparently identical to P-450p, and, in addition, have recently found a homologous cDNA (P-450PCN2) that differs from P-450PCN1 in its deduced NH₂-terminal amino acid sequence (Gonzalez et al., 1986). However, no laboratory has isolated more than one P-450p-related protein from a single species. Some apparently homogeneous preparations may, in fact, be mixtures of closely related polypeptides that cross-react with polyclonal antibodies.

In the present study, monoclonal antibodies that recognize

selectively individual PCN-inducible hepatic cytochromes P-450 were prepared. On the basis of analyses of rat liver microsomes with these probes, it is concluded that rat liver contains multiple immunochemically similar P-450p-related proteins that display separate regulatory characteristics.

MATERIALS

The polychlorinated and polybrominated biphenyl isomers, 2,4,2',4'-tetrachlorobiphenyl and 2,4,5,2',4',5'-hexabromobiphenyl, were generous gifts from Dr. Stephen Safe and Dr. Stephen Aust, respectively. Chlordane and trans-nonachlor were gifts from Velsicol (Chicago, IL). Aroclor 1254 was obtained from Monsanto Co. (St. Louis, MO) and SKF-525A was provided by Smith, Kline, and French (Philadelphia, PA). Triacetyloleandomycin was a gift of Pfizer Laboratories (Brooklyn, NY) and PCN was provided by the Upjohn Co. (Kalamazoo, MI). Phenobarbital sodium was purchased from Amend Drug and Chemical Co. (Irvington, NJ); isosafrole from Fluka (New York, NY); HPLC-grade isopropanol from Burdick and Jackson (Muskegan, MI); spironolactone, trans-stilbene oxide, chlorpromazine hydrochloride, phenothiazine, diphenylhydantoin, metyrapone, dexamethasone, clofibrate, and corn oil from Sigma (St. Louis, MO); peroxidase-conjugated anti-mouse IgG, rabbit anti-goat IgG, and goat peroxidase anti-peroxidase from Miles Scientific (Elkhart, IN); nitrocellulose paper from Bio-Rad (Richmond, CA); and diaminobenzidine tetrahydrochloride from Pfaltz and Bauer (Stamford, CT). All other chemicals used were of reagent grade or better.

METHODS

Animals and Treatments. Sprague-Dawley rats (100-150 g, Flow Laboratories, Dublin, VA, USA) were housed in pairs in wire bottom cages and given unlimited access to standard lab chow and water, unless otherwise indicated. All chemicals were dissolved in corn oil except chlorpromazine, phenobarbital, and isopropanol, which were dissolved or diluted in water, metyrapone, which was dissolved in 0.9% NaCl and triacetyloleandomycin, which was added to powdered chow. Single intraperitoneal injections of 2,4,2',4'-tetrachlorobiphenyl (500 μ moles/kg) and 2,4,5,2',4',5'-hexabromobiphenyl (500 μ moles/kg) were given four and seven days, respectively, prior to microsome preparation. A single intraperitoneal injection was also given for Aroclor 1254 (300mg/kg) four days prior to microsome preparation. Daily intraperitoneal injections were given for spironolactone (100mg/kg), trans-stilbene oxide (300mg/kg), isosafrole (150mg/kg), chlorpromazine (40mg/kg), phenothiazine (100mg/kg), PCN (300mg/kg), diphenylhydantoin (50mg/kg), trans-nonachlor (46mg/kg), and chlordane (42mg/kg), each for four days and SKF-525A (80mg/kg) and clofibrate (400mg/kg), each for three days. Dexamethasone (300mg/kg) and isopropanol (15mmoles/kg) were given by oral gavage daily for four days and for one day, respectively; phenobarbital (0.1%) was administered in drinking water for 6 days; triacetyloleandomycin (1% w/w) was fed in powdered chow for 10 days; and metyrapone (500mg/kg) was given as a single intraperitoneal injection one day prior to microsome preparation.

Each animal was fasted overnight and killed by decapitation. The

liver was perfused retrograde with cold phosphate buffered saline (PBS), pH 7.4, excised and homogenized, and microsomes were isolated by differential centrifugation as previously described (Elshourbagy & Guzelian, 1980). The concentration of total CO-binding cytochrome P-450 in the microsomal preparations and in the purified proteins was determined by difference spectroscopy as described by Omura and Sato, 1964. Microsomal protein concentrations were determined colorometrically (Schacterle & Pollack, 1973).

Rat cytochrome P-450p (Wrighton et al., 1985a), rabbit cytochrome LM3c (Koop et al., 1981), and human cytochrome HLP (Watkins et al., 1985) were purified according to the indicated published procedures.

Preparation of Antibodies. Five virus-free female Balb/c mice (15-18 g, Charles River Labs, Raleigh, NC) were each injected intraperitoneally with 50 μ g of purified P-450p in a 50mM potassium phosphate, 50mM EDTA, 20% glycerol buffer combined with an equal volume of Freund's complete adjuvant in a total volume of 0.2ml, followed 21 days later by a 20 μ g boost. Six days after the boost, blood samples from the mice were pooled and serum was prepared and assayed for the presence of antibody by enzyme linked immunosorbent assay (ELISA). A final 20 μ g intravenous boost was performed 3 days prior to fusion. Spleen cells from one immunized mouse were fused with P3-X63-Ag8 multiple myeloma cells and the resulting hybridoma were expanded in vitro. Limiting dilutions were carried out to isolate individual clones. These were tested for antibody production by ELISA, allowed to expand, and then injected intraperitoneally into female Balb/c mice (5 X 10⁶ cells per mouse) which had been primed with pristane (0.5ml intraperitoneally) two weeks previously. The resulting ascitic fluid

was collected 9 to 14 days later, centrifuged to remove cells, and then stored at -20° C. The second monoclonal antibody used in these studies, termed 13-7-10, was prepared by Dr. Kremers as described elsewhere (Beaune *et al.*, 1985).

A polyclonal anti-P-450p IgG raised in a goat was prepared as previously described (Elshourbagy *et al.*, 1981). In some immunoblot experiments absorbed anti-P-450p was used. Briefly, liver microsomes prepared from a 3-methylcholanthrene-treated male rat were solubilized with cholate and bound to cyanogen bromide-activated Sepharose-4B (15mg microsomal protein/ml Sepharose-4B) (Cautreccasas, 1970; March *et al.*, 1974). The IgG fraction prepared from serum of a goat immunized with P-450p was incubated overnight with the immobilized microsomes (100mg IgG/ml of Sepharose-4B). The unbound IgG was collected and subjected to a second immunoabsorption step (25mg IgG/ml of Sepharose-4B) with liver microsomes prepared from an untreated female rat bound to the Sepharose matrix.

Immunoblot Analysis. Quantitative analyses of immunoblots were performed as described previously (Schuetz *et al.*, 1984). Briefly, microsomal proteins were electrophoretically separated in 10% sodium dodecylsulfate-polyacrylamide slab gels. After the initial two hours of electrophoresis, a different amount of purified protein standard was added to each well. Electrophoresis was then continued for an additional one to two hours. The resolved proteins were electrophoretically transferred to nitrocellulose paper and non-specific protein binding sites were blocked by an overnight incubation of the nitrocellulose paper in PBS containing 3% bovine serum albumin and 10% calf serum. After a one hour exposure to the monoclonal

antibody, the blots were washed with cold PBS, incubated with peroxidase-conjugated anti-mouse IgG for 30 minutes, and washed again with PBS. The immunoreactive proteins were visualized with the use of diaminobenzidine in 0.006% hydrogen peroxide. The blots were scanned with a Ziess densitometer to determine the integrated optical density of each protein band. The amount of immunoreactive protein present in each microsomal sample was calculated from the linear portion of a standard curve derived from the densities of the purified proteins on the same blot. Qualitative immunoblots developed with goat anti-P-450p IgG required a 30 minute exposure to rabbit anti-goat IgG and a 30 minute exposure to goat peroxidase anti-peroxidase with intervening PBS washes.

RESULTS AND DISCUSSION

Monoclonal antibody 1G8 was selected because this unique probe reacted with purified rat P-450p on immunoblots but did not react with the purified homologous proteins, rabbit cytochrome LM3c (Wrighton et al., 1985a) or human cytochrome HLP (Watkins et al., 1985) (Fig. 1). Nevertheless, these three homologous proteins are immunochemically related in being recognized by polyclonal antibodies raised against either P-450p (Fig. 2, top) or LM3c (Watkins et al., 1985). Immunoblot analyses of liver microsomes from untreated male rats developed with 1G8 revealed two narrowly separated proteins (Fig. 1). The more slowly migrating of the two 1G8-reactive proteins in untreated males had the same mobility as did purified P-450p (Fig. 3). In contrast, no proteins were detected in liver microsomes isolated from untreated female rats when immunoblotting was performed under conditions of complete transfer (Schuetz et al., 1984) (Fig. 1). When these conditions were exceeded, trace amounts of 1G8-reactive protein were observed indicative of very low level expression of P-450p in livers of untreated female rats (see Appendix, figure 1).

Next, a second monoclonal antibody, 13-7-10, was characterized. This antibody reacts specifically with HLP, a glucocorticoid-inducible human liver cytochrome P-450 (Molowa et al., 1986). The reactivity of this monoclonal antibody was the opposite of 1G8 in that 13-7-10 specifically recognized purified rabbit LM3c and human HLP, but did not recognize purified P-450p (Fig. 1). Immunoblots of rat liver microsomes developed with the 13-7-10 antibody revealed a reactive

protein in both untreated males and untreated females (Fig. 1). A consistent finding was that the 13-7-10-reactive protein in females migrated more slowly than did the 13-7-10-reactive protein in males (Fig. 1). Thus, two distinct 13-7-10-reactive proteins can be distinguished on the basis of their mobilities and sex-specificities. One or both of these proteins may be inducible because dexamethasone treatment increased the amount of total 13-7-10-reactive microsomal protein in female rats (Fig. 1).

The possibility that the microsomal proteins recognized by these monoclonal antibodies are only coincidentally related to P-450p cannot be excluded. However, polyclonal antiserum directed against P-450p reacted not only with all three purified homologous proteins, P-450p, LM3c, and HLP, but also with a pair of closely migrating, but separable proteins, in rat liver microsomes from an untreated male rat and a second pair in microsomes from an untreated female rat (Fig. 2). When the antiserum was absorbed first against immobilized rat liver microsomes prepared from 3-methylcholanthrene-treated males and then against untreated females, the resulting antibody still reacted strongly with purified P-450p, but only weakly with LM3c and HLP and not at all with microsomes from untreated female rats (Fig. 2). Thus, it appears that LM3c, HLP, and the 13-7-10-reactive protein present in untreated female rats share one or more epitopes that are recognized by neither the 1G8 monoclonal antibody nor the absorbed anti-P-450p IgG.

Finally, when portions of the same immunoblot were developed with the anti-P-450p antiserum or with one of the two monoclonal antibodies, the pair of 1G8-reactive proteins in untreated male rats co-migrated exactly with the proteins identified by the polyclonal antiserum (Fig.

4). Likewise, the proteins identified by the 13-7-10 monoclonal antibody (one in untreated males and one in untreated females) exhibited electrophoretic mobility identical to those recognized by the polyclonal antiserum (Fig. 4). Therefore, the proteins reacting with the unabsorbed polyclonal antibody and the monoclonal antibodies are probably the same. There is no evidence to suggest that the faster migrating female protein which was recognized only by the unabsorbed polyclonal antibody is related to the P-450p family. Therefore, it is concluded that there are at least three related proteins expressed in the livers of untreated rats: P-450p, and the male- and female-specific 13-7-10-reactive proteins. It is possible that the faster migrating of the two 1G8-reactive proteins in untreated males and the male-specific 13-7-10-reactive protein are the same, since both proteins exhibit similar electrophoretic mobilities.

The next series of experiments investigated the effects of treatment of female rats with inducers of P-450p on the amounts of 1G8- and 13-7-10-reactive proteins as determined by quantitative immunoblot analyses of liver microsomes. Because it was not possible to sufficiently separate and individually quantitate the pairs of proteins in induced rats, the results are expressed as the total 1G8- or total 13-7-10-reactive proteins. These studies utilized only one dose of each compound at a level reported to be maximal for induction of CO-binding hemoprotein. TAO induced the 1G8-reactive proteins to the greatest extent (>300-fold increase over the minimum detectable value) (Table 1). This is consistent with the results of previous experiments in which P-450p was quantitated by a polyclonal anti-P-450p IgG preparation (Wrighton et al., 1985b). Other strong inducers of

1G8-reactive proteins were dexamethasone (90-fold), chlordane (85-fold), PCN (79-fold), trans-nonachlor (63-fold), SKF-525A (41-fold), spironolactone (39-fold), metyrapone (32-fold), and 2,4,2',4'-tetrachlorobiphenyl (20-fold) (Table 1). In contrast, TAO only moderately induced the 13-7-10-reactive proteins (21-fold increase) compared to dexamethasone (49-fold increase) (Table 1). Most striking, however, was the relative lack of induction of 13-7-10-reactive proteins by chlordane and trans-nonachlor, both of which were strong inducers of the 1G8-reactive proteins (Table 1). Similarly, SKF-525A, spironolactone, and metyrapone were strong inducers of the 1G8-reactive proteins but relatively poor inducers of the 13-7-10-reactive proteins (Table 1). In addition, 2,4,2',4'-tetrachlorobiphenyl only induced 1G8-reactive proteins (Table 1). Weaker inducers of the 1G8-reactive proteins included isosafrole, phenobarbital, trans-stilbene oxide, and Aroclor 1254 (Table 1). Each of these compounds was a superior inducer of the 1G8-reactive proteins as compared with the 13-7-10-reactive proteins (Table 1). Phenothiazine, chlorpromazine, and isopropanol exclusively induced the 1G8-reactive proteins whereas 2,4,5,2',4',5'-hexabromobiphenyl, albeit a weak inducer, produced similar increases in the 1G8- and 13-7-10-reactive proteins (Table 1). The responses of the 1G8- and 13-7-10-reactive proteins to diphenylhydantoin and clofibrate were only marginal increases (Table 1).

This laboratory previously demonstrated that there is a striking, qualitative difference in the regulatory control of P-450p-related proteins between species (Wrighton et al., 1985b). For example, rifampicin, the most efficacious inducer of P-450 LM3c in the rabbit, does not at all induce P-450p in the rat. Conversely, PCN, an inducer

of rat P-450p, is not an inducer of P-450 LM3c in the rabbit. In this study, using quantitative analyses, a striking difference was found in the profile of induction among apparently related proteins within a single species. For example, the organochlorine pesticides chlordane and trans-nonachlor, which strongly induced the 1G8-reactive proteins were weak inducers of the 13-7-10-reactive proteins (Table 1). Indeed, 2,4,2',4'-tetrachlorobiphenyl appeared to exclusively induce 1G8-reactive proteins (Table 1). Although preferential induction of cytochrome P-450 forms within a family has been demonstrated (Ryan et al., 1980; Thomas et al., 1983), an example of induction of one (or more) isozymes within an inducible family to the exclusion of other family members has not been previously reported. Thus it may be concluded that although the 13-7-10-reactive proteins are related to P-450p, they are subject to quantitatively and qualitatively distinct control.

Gonzalez et al. have reported differential regulation of two mRNA's encoding members of the PCN family (Gonzalez et al., 1986). They found that P-450PCN1 mRNA was undetectable in both untreated male and female rats but was readily inducible by PCN and dexamethasone. The homologous P-450PCN2 mRNA was present in untreated male and female rats, was inducible by phenobarbital, but was refractory to induction by PCN and dexamethasone. The reported expression of P-450PCN2 mRNA does not correspond to that of the immunoreactive proteins we have described because none of the 1G8- or 13-7-10-reactive proteins are present in both males and females and both groups of proteins are readily inducible by PCN or dexamethasone (Table 1). The resolution of these discrepancies awaits more complete characterization of the

multiple mRNA and protein species.

The immunoquantitation results provide a basis for ruling out the possibility that the 13-7-10-reactive proteins might be previously characterized rat liver cytochromes. For example, cytochromes P-450b and P-450e are elevated 20- to 30-fold in phenobarbital treated rats (Waxman, 1986) which is inconsistent with the weak response of the 13-7-10-reactive proteins to phenobarbital (Table 1). Likewise, it is unlikely that 13-7-10 recognizes cytochromes P-450c and P-450d, which are elevated 30-fold by Aroclor and 20-fold by isosafrole treatments, respectively (Waxman, 1986) (see Table 1). Cytochrome P-450 PB-C (P-450 PB-1) (Guengerich et al., 1982; Waxman, 1986), which is induced by neither PCN nor Aroclor (Guengerich et al., 1982) can also be ruled out. Finally, rat liver cytochromes P-450a, P-450f, P-450g, P-450h, P-450i (Ryan et al., 1982; Ryan et al., 1984a; Ryan et al., 1984b), and P-450 UT-H (Larrey et al., 1984) are proteins which are not significantly induced and actually may decrease following treatment with phenobarbital, PCN, isosafrole, and Aroclor (Guengerich et al., 1982).

Vlasuk et al., 1982, used two dimensional gel electrophoresis to tentatively identify as P-450p a polypeptide that was barely detectable in untreated rats, was induced by PCN, isosafrole, SKF-525A, Aroclor 1254, and trans-stilbene oxide, but was not induced by chlordane. This laboratory recently identified a protein that had mobility on two dimensional gel electrophoresis identical to that of P-450p, was recognized by polyclonal anti-P-450p IgG, and was induced by chlordane (Schuetz et al., 1986). Thus the polypeptide identified by Vlasuk and co-workers may represent a distinct member of a PCN family (Schuetz et al., 1986). The exact relationship between the latter protein and the

P-450p-related proteins we have identified in the present report remains unclear since SKF-525A, isosafrole, and chlordane elevate the 13-7-10-reactive proteins to approximately the same extent (about 5-fold above the minimum detectable amount) and Aroclor 1254 and trans-stilbene oxide were only marginally efficacious inducers of 13-7-10-reactive proteins (Table 1).

It was observed that 13-7-10-reactive proteins were only weakly induced by TAO (Table 1). This might reflect differences in substrate specificity of the 13-7-10-reactive proteins because the dramatic induction of 1G8-reactive P-450p in TAO-treated rats is associated with conversion of the drug by P-450p to a metabolite that binds P-450p specifically (Wrighton et al., 1985a). In contrast, an agent has yet to be identified that elevates 13-7-10-reactive proteins more than the 1G8-reactive proteins. The most effective in this regard is 2,4,5,2',4',5'-hexabromobiphenyl, which elevated these proteins to a similar extent. Although no attempt was made to determine dose-response relationships for the inducers, structural differences among the polyhalogenated biphenyls may account for the apparent preferential or selective induction of the two types of immunoreactive proteins (Table 2). Indeed, this laboratory recently demonstrated that induction of P-450p and P-450b/e by polychlorinated biphenyl isomers involves different structure-activity relationships (Schuetz et al., 1986).

In summary, based on immunoblot analyses of rat liver microsomes developed with the antibody preparations described, at least two heretofore unrecognized proteins distinct from but related to P-450p have been identified. Final proof that these proteins are products of separate genes will require their purification, structural elucidation,

and assessment of their immunoreactivity. Nevertheless, specific monoclonal antibodies provide strong albeit indirect evidence to add to the growing recognition that there is a family of "PCN inducible-cytochromes P-450" expressed in rat liver. These proteins appear to be discoordinately regulated. They are under complex control by factors associated with gender and are influenced to different extents by many structurally dissimilar exogenous compounds. The monoclonal antibodies described will be most helpful in eventually achieving a complete structural description of this unique family of proteins and the mechanisms of their regulation.

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Table 1

Quantitation with monoclonals 1G8 and 13-7-10 of immunoreactive cytochromes P-450 in rats treated with inducers. Female rats were treated and liver microsomes prepared and analyzed by quantitative immunoblotting as described in Methods. Results are expressed as densitometric units per microgram of microsomal protein and the corresponding fold increase over the lowest detectable value^a. Shown are the mean \pm standard deviation of values obtained from three individual rats.

<u>Inducer</u>	<u>1G8-reactive</u>	<u>Fold Increase</u>	<u>13-7-10-reactive</u>	<u>Fold Increase</u>
Control	<0.007	-	<0.007	-
TAO ^b	2.230 \pm 562	319	0.147 \pm .040	21
DEX	0.631 \pm 121	90	0.341 \pm .063	49
CD	0.594 \pm .129	85	0.034 \pm .012	5
PCN	0.555 \pm .049	79	0.147 \pm .029	21
TNC	0.441 \pm .065	63	0.028 \pm .004	4.0
SKF	0.284 \pm .090	41	0.043 \pm .006	6.1
SLN	0.270 \pm .071	39	0.044 \pm .012	6.3
MTP	0.223 \pm .034	32	0.033 \pm .013	4.7
PCB	0.141 \pm .040	20	<0.007	-
ISF	0.110 \pm .039	16	0.033 \pm .022	4.7
PB	0.096 \pm .021	14	0.049 \pm .018	7.0
TSO	0.081 \pm .017	12	0.012 \pm .005	1.7
ARO	0.078 \pm .022	11	0.009 \pm .022	1.3
PTZ	0.050 \pm .019	7	<0.007	-
PBB	0.040 \pm .004	5.7	0.027 \pm .017	3.9
CLO	0.039 \pm .008	5.6	0.009 \pm .002	1.3
DPH	0.022 \pm .008	3.1	0.009 \pm .002	1.3
CPZ	0.019 \pm .006	2.7	<0.007	-
ISO	0.012 \pm .005	1.7	<0.007	-

Table 1 (cont'd.)

^aDetection of 1G8- and 13-7-10-reactive proteins in microsomes isolated from untreated female rats requires analysis of samples that exceed the conditions established for quantitative electrophoretic transfer. The above values for fold induction are calculated as increases over the lowest detectable value (.007 units/ug) and thus underestimate the magnitude of induction.

^bAbbreviations used are: TAO, triacetyloleandomycin; DEX, dexamethasone; CD, chlordane; PCN, pregnenolone-16 α -carbonitrile; TCN, trans-nonachlor; SKF, SKF-525A; SLN, spironolactone; MTP, metyrapone; PCB, 2,4,2',4',-tetrachlorobiphenyl; ISF, isosafrole; PB, phenobarbital; TSO, trans-stilbene oxide; ARO, arochlor; PTZ, phenothiazine; PBB, 2,4,5,2',4',5'-hexabromobiphenyl; CLO, clofibrate; DPH, diphenylhydantoin; CPZ, chlorpromazine; ISO, isopropanol.

Figure 1. Immunoblots of purified cytochromes P-450 and rat liver microsomes. Protein samples were electrophoretically resolved in polyacrylamide slab gels, transferred to nitrocellulose paper, and then developed with either monoclonal 1G8 (top) or 13-7-10 (bottom) as described in "Methods". Shown are 1 μ g each of purified rat P-450p, rabbit LM3c, and human HLP; 50 μ g of microsomal protein from untreated rats, male (M) and female (F); and 10 μ g of microsomal protein from a dexamethasone-treated female rat (DEX).

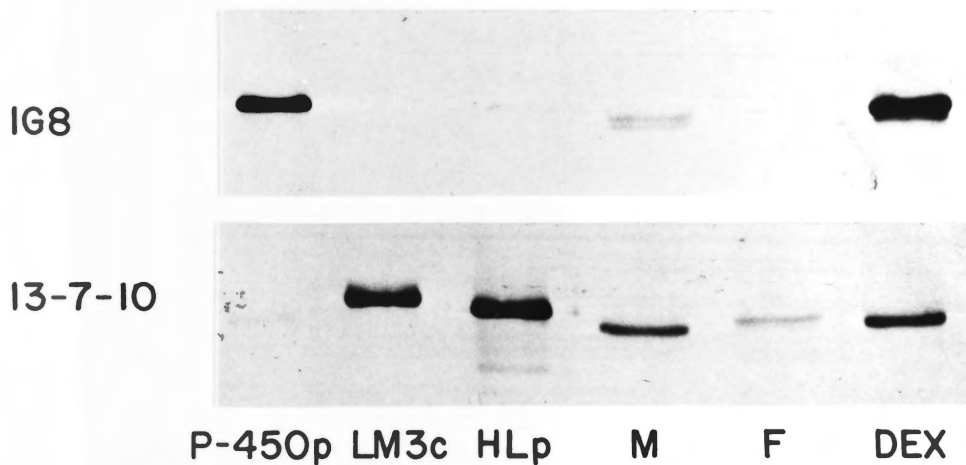


Figure 2. Specificity of anti-P-450p antibody before and after immunoabsorption. Purified cytochromes P-450 and rat liver microsomes were analyzed by immunoblots, as described in "Methods", developed with either polyclonal anti-P-450p serum (top) or anti-P-450p antibody after 2X-immunoabsorption (bottom). Shown are 0.5 μ g each of purified rabbit LM3c, human HLp and rat P-450p, and 50 μ g of microsomal protein from untreated male (M) and female (F) rats.

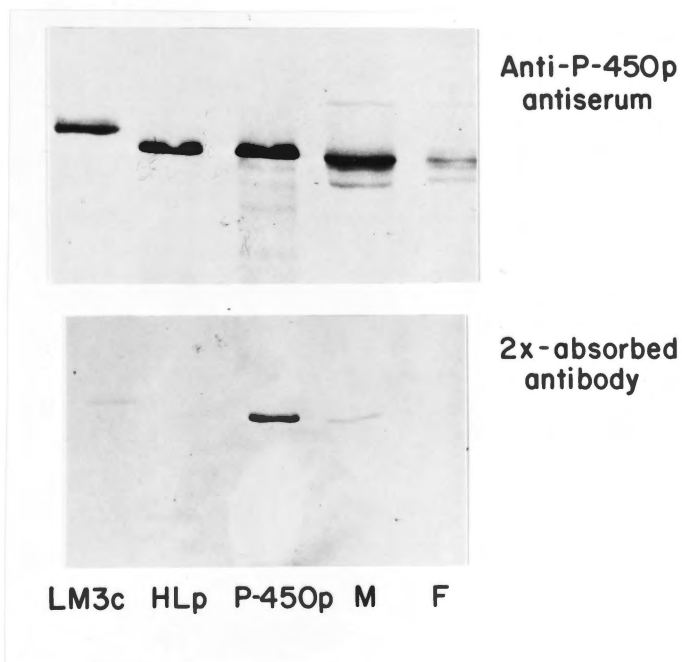


Figure 3. Relative electrophoretic mobility of 1G8-reactive microsomal proteins with purified P-450p. Immunoblot of purified rat P-450p (0.5 μ g) and of microsomal proteins from an untreated male rat (M) (50 μ g) and from a dexamethasone-treated female rat (1 μ g) (DEX) developed with monoclonal antibody 1G8.

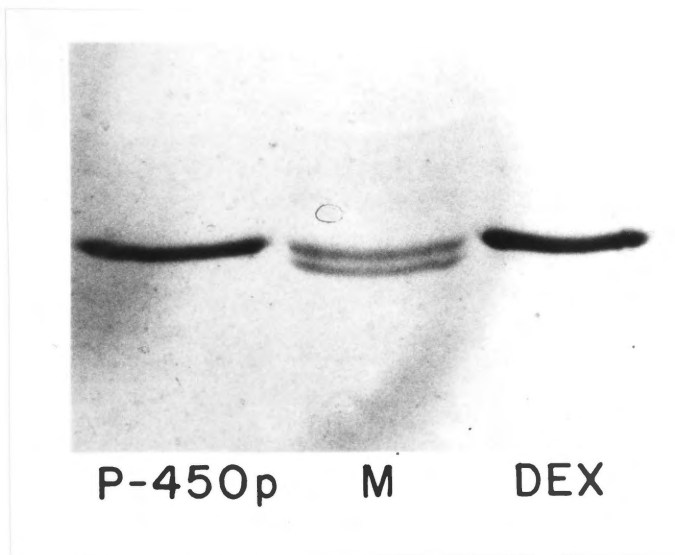
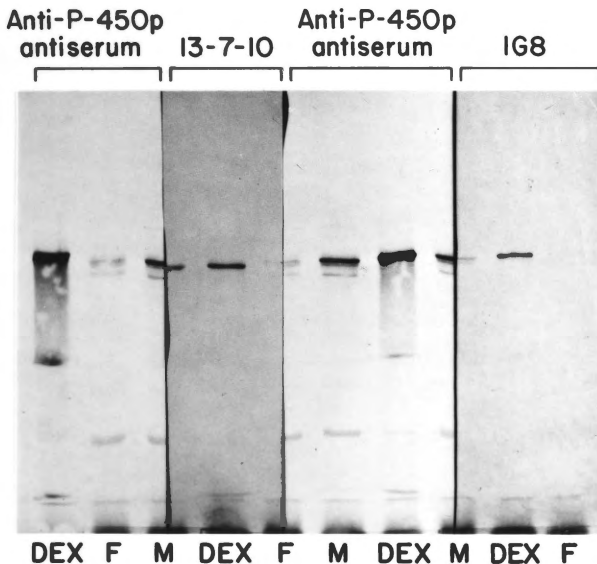


Figure 4. Analyses of rat liver microsomes by immunoblots developed with various antibodies. Shown are microsomal protein samples (50 μ g) from untreated male (M) and female (F) rats and microsomal protein samples (5 μ g) from a dexamethasone-treated female rat (DEX). Prior to development, the immunoblot was cut into strips bisecting the lanes containing microsomal protein samples from untreated rats. Each strip was then developed with the indicated antibody, as described in "Methods". The dye front is shown to demonstrate proper realignment of the nitrocellulose paper.



CHAPTER TWO

Co-induction of Multiple Hepatic
Cytochrome P-450 Proteins and mRNAs in Rats Treated
With Imidazole Antimycotic Agents.

ABSTRACT

To characterize the molecular basis by which imidazole antimycotic drugs increase cytochrome P-450, the effects of treating rats with clotrimazole, miconazole, or ketoconazole on expression of three forms of the hepatic cytochromes P-450 (P-450p, P-450b/e, and P-450j) were examined. From measurements of liver microsomal catalytic activities (demethylation of erythromycin, benzphetamine, or N-nitrosodimethylamine), microsomal content of immunoreactive cytochrome P-450 protein, and the amounts of liver RNA hybridizing to cloned P-450 cDNAs, it was 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 induced modestly by clotrimazole and miconazole, but not at all by ketoconazole. In contrast, ethanol-responsive P-450j was induced by ketoconazole but not by clotrimazole or miconazole. In several instances, treatment of rats with antifungal drugs resulted in accumulation of P-450 protein which significantly exceeded the corresponding increase in P-450 mRNA. Specifically, clotrimazole and ketoconazole disproportionately elevated P-450p and P-450j protein, respectively, whereas P-450b/e protein accumulated in excess of its mRNA following treatment with either clotrimazole or miconazole. From these results, it was concluded that imidazole antifungal drugs differentially modulate the expression of at least three distinct gene families of rat hepatic cytochromes P-450 by separate mechanisms involving accumulation of cytochromes P-450 mRNA and hemeprotein.

INTRODUCTION

Oxidative biotransformation of lipophilic compounds is catalysed by membrane-bound heme proteins 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 (Lu & West, 1980). 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 (Whitlock, 1986). For example, barbiturates such as phenobarbital, polycyclic aromatic hydrocarbons such as β -naphthoflavone, glucocorticoids and anti-glucocorticoids such as pregnenolone-16 α -carbonitrile (PCN), and ketones and alcohols such as ethanol are examples of four different classes of agents that selectively induce one of four different families of cytochrome P-450.

Recently, N-substituted imidazole compounds have been shown to increase the total amount of cytochrome P-450 in the liver (Lavrijsen et al., 1986; Pershing et al., 1986; Ritter & Franklin, 1987). Several N-substituted imidazole derivatives are well known to be potent inhibitors of oxidative metabolism (Sheets et al., 1986). These clinically useful antifungal agents interrupt synthesis of the major fungal sterol, ergosterol, at the cytochrome P-450-mediated step of lanosterol 14 α -demethylation (Van den Bossche et al., 1983). When one of these drugs, such as clotrimazole, miconazole, or ketoconazole, is

administered to rats, liver microsomal oxidation of typical cytochrome P-450 substrates including erythromycin, aminopyrene, N,N-dimethylaniline, or p-nitroanisole is increased (Lavrijsen et al., 1986; Pershing et al., 1986; Ritter & Franklin, 1987). Analysis of the profile of induced drug oxidizing activities suggests that clotrimazole may be a steroid-like inducer of cytochrome P-450 (Pershing et al., 1986; Ritter & Franklin, 1987), and miconazole may possess both steroid and phenobarbital inducing characteristics (Lavrijsen et al., 1986; Ritter & Franklin, 1987). However, ketoconazole induces a distinct profile of microsomal activities that do not resemble those from phenobarbital, steroid, or methylcholanthrene-treated rats (Lavrijsen et al., 1986).

In the present study, the identity of the cytochrome P-450 isozymes regulated by these N-substituted imidazoles was investigated not only by measuring microsomal drug oxidizing activities, but also by analyzing liver microsomes on immunoblots developed with specific antibodies directed against the major form(s) of cytochrome P-450 induced by glucocorticoids (P-450p), by phenobarbital (P-450b/e), or by ethanol (P-450j). In addition, Northern blot analyses of liver RNA were carried out to determine the amounts of mRNA hybridizable to cloned cDNA probes to P-450p, P-450b/e, and P-450j. The results reveal that P-450p is the major cytochrome induced by clotrimazole, miconazole, and ketoconazole, and that the former two also induce cytochrome P-450b/e, whereas only ketoconazole induced P-450j. Moreover, there was a lack of proportionality between induction of immunoreactive P-450 protein as compared to increases in hybridizable P-450 mRNA suggesting that these antimycotics selectively induce some cytochromes P-450 by effects on both mRNA and protein metabolism.

MATERIALS

Triacetyloleandomycin (TAO) and ketoconazole were gifts from Pfizer Laboratories (Brooklyn, NY) and Janssen Pharmaceutica (Piscataway, NJ), respectively. Erythromycin and d-benzphetamine were purchased from the Upjohn Co. (Kalamazoo, MI); phenobarbital sodium from the Amend Drug and Chemical Co. (Irvington, NJ); clotrimazole, N-nitrosodimethylamine, dexamethasone, β -naphthoflavone, and miconazole nitrate from Sigma Chemical Co. (St. Louis, MO). 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 anti-peroxidase from Miles Scientific (Elkhart, IN) and peroxidase anti-mouse IgG, peroxidase anti-rabbit IgG, and rabbit anti-goat IgG from ICN ImmunoBiologicals (Lisle, IL). All other reagents were of the finest grade commercially available.

METHODS

Animals and treatments- Sprague-Dawley rats (175 - 225 grams, Flow Labs, Dublin, VA) were housed in pairs and given unlimited access to food and water. Each compound was suspended in corn oil (except phenobarbital, which was dissolved in 0.9% sodium chloride) and was administered to female rats by oral gavage (except phenobarbital and β -naphthoflavone, which were given by intraperitoneal injection). Miconazole (150mg/kg), ketoconazole (150mg/kg), clotrimazole (100mg/kg or 150mg/kg), phenobarbital (80mg/kg) and β -naphthoflavone (80mg/kg) were each administered daily for three days. Dexamethasone (300mg/kg), and TAO (480mg/kg) were given daily for three and five days, respectively. Vehicle-treated male and female rats were given corn oil (10ml/kg) for three days. Animals were fasted overnight and then killed by decapitation 24 hours after the final treatment unless otherwise noted. Each liver was perfused with iced phosphate buffered saline (PBS), pH 7.4, and then was excised and homogenized with a motor-driven teflon pestle in a buffer containing 100mM Tris (pH 7.4) 100mM KCl, 1mM EDTA, and 20 μ M butylhydroxytoluene. Microsomes were isolated by differential centrifugation as previously described and stored immediately at -70°C (Elshourbagy & Guzelian, 1980). Total CO-binding heme protein content of each microsomal sample was determined as the dithionite reduced CO-difference spectrum (Omura & Sato, 1964). The sample cuvette was saturated with CO for 60 seconds and sequential difference spectra were recorded until the absorbance at 450nm reached a maximum. Microsomes from TAO-treated rats were decomplexed with

$K_3Fe(CN)_6$ prior to the addition of carbon monoxide (Wrighton et al., 1985a). Microsomal protein concentrations were determined colorimetrically (Schacterle & Pollack, 1973).

Isolation of Purified Proteins and Antibodies- Rat liver cytochromes P-450p (Wrighton et al., 1985b), P-450b (Waxman & Walsh, 1982), and P-450j (Ryan et al., 1985) were purified according to indicated published procedures. A monoclonal antibody directed against purified P-450p was prepared and characterized as described (see Chapter 1). The polyclonal antibody directed against purified P-450b was raised in a goat as described (Elshourbagy et al., 1981) and then processed by sequential immunoabsorptions, first against liver microsomes from β -naphthoflavone-treated rats, and then against microsomes from untreated male rats. The polyclonal antibody directed against purified P-450j was raised in rabbits and characterized as indicated (Thomas et al., 1987).

Microsomal N-demethylase Assays- Microsomal benzphetamine and N-nitrosodimethylamine N-demethylase activities were determined essentially as described (Guengerich, 1982). Briefly, 1.0 mg of microsomal protein from control or treated rats was incubated at 37° C in a 100mM potassium phosphate buffer containing either 1.0 mM d-benzphetamine or 1.0 mM N-nitrosodimethylamine along with glucose-6-phosphate and glucose-6-phosphate dehydrogenase to generate NADPH. Erythromycin N-demethylase activity was measured as previously reported (Wrighton et al., 1985a) with erythromycin present at a final concentration of 0.4 mM and NADPH (40 mM final concentration) added exogenously. Enzymatic production of formaldehyde formed was determined colorimetrically using a Nash reagent (Nash, 1953).

Microsomes from TAO-treated rats were decomplexed with $K_3Fe(CN)_6$ prior to the initiation of the N-demethylase reactions.

Immunoblot Analyses- Liver microsomes were resolved in polyacrylamide slab gels and blotted onto nitrocellulose filters under conditions described previously (Schuetz et al., 1984; and see Chapter 1). 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 minute exposure to the appropriate antibody. Exposure of blots to anti-P-450p antibody or anti-P-450j antibody was followed by a 30 minute incubation with peroxidase-conjugated anti-mouse or anti-rabbit IgG, respectively. Immunoblots developed with anti-P-450b antibody required sequential 30 minute incubations with rabbit anti-goat IgG and peroxidase goat anti-peroxidase. Nitrocellulose filters were washed thoroughly with PBS between successive antibody incubations. Immunoreactive proteins were visualized with N,N-diaminobenzidine in 0.006% H_2O_2 . Quantitative immunoblot analyses included the appropriate purified cytochrome P-450 (0.2 to 2.0 pmoles for purified P-450p and P-450b, and 1.0 to 6.0 pmoles for P-450j). The integrated density of each immunoreactive band for both standards and samples was quantitated with a Zeiss densitometer as described (see Chapter 1).

Isolation and Analyses of RNA- The P-450p (Wrighton et al., 1985a), P-450b/e (Adesnik et al., 1981), and P-450j (Wrighton et al., 1986) cDNA probes were isolated and characterized in the indicated references. Total liver RNA from control or treated rats was isolated from a 0.5 g portion of liver using the guanidine isothiocyanate method (Elshourbagy et al., 1981; Deeley et al., 1977). Northern blot

analyses were carried out as previously described (Wrighton et al., 1985a). 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 -labelled cDNA probes as described previously (Schuetz et al., 1986). Hybridization of the labelled cDNA probes to filter-bound RNA was visualized by autoradiography. Identical hybridization conditions were utilized for quantitation, except that RNA samples were diluted and applied directly to nitrocellulose filters using a Schleicher and Schuell slot blot apparatus according to the manufacturers specifications. For each treatment group, a range of RNA (from 0.05 to 30 μg) was analyzed. Autoradiography and scanning densitometry revealed a linear relationship between optical density of the hybridization signal and the amount of RNA analyzed. A slope was determined for each animal and was compared to the slope representing hybridizable mRNA from a control female rat analyzed on the same filter.

Statistical methods- Two-tailed student t tests were performed on unpaired sample means and the significance determined at two levels, $p \leq 0.01$ and $p \leq 0.05$.

RESULTS

Effect 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 in previous reports (Lavrijsen et al., 1986; Ritter & Franklin, 1987), although the maximum tolerated dose of each of these compounds has not been reported. Preliminary studies demonstrated that clotrimazole administered at 150mg/kg daily for three days produced a remarkable increase (5.1-fold) in total CO-binding cytochrome P-450 (Figure 1). This induction was even greater than that produced by triacetyloleandomycin (TAO) (Figure 1), a macrolide antibiotic inducer of P-450p which, until now, was the most efficacious inducer of rat liver cytochrome P-450 previously reported (Wrighton et al., 1985b; and Chapter 1). Treatment of rats with the maximum tolerated dose (150mg/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 dexamethasone (2.3-fold) or phenobarbital (1.9-fold) (Figure 1). It was observed that only 2 to 3 minutes was required to achieve the peak absorbance at 450nm after CO saturation of the sample cuvette, except for samples from clotrimazole-treated rats, which required 16 to 24 minutes (see Appendix, figure 2). Others have reported a slowly developing difference spectrum, indicative of competition between CO and a compound tightly bound to heme (Ritter & Franklin, 1987; Yoshida & Aoyama, 1987). To test this idea, female rats were treated with a reduced dose of clotrimazole (100mg/kg daily

for 3 days) and liver microsomes were isolated 42 hours (instead of the routine 24 hours) after the final clotrimazole treatment. 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 minutes before the CO-difference spectrum to reached a maximum.

Effect of Treatments on Microsomal N-demethylase Activities-

Erythromycin N-demethylase (ERMD), a microsomal function known to be catalysed by P-450p (Wrighton et al., 1985b) was increased 7.3-fold above control values by clotrimazole treatment (Table 1). To reduce the interference by a residual competitor, (see preceding paragraph), microsomes were isolated 42 hours after the last clotrimazole treatment for ERMD measurements. The magnitude of induction of ERMD by clotrimazole was similar to that produced by TAO and dexamethasone, two known inducers of P-450p (Table 1) (Wrighton et al., 1985a; see also Chapter 1). Miconazole treatment elevated ERMD activity to a lesser degree (2.2-fold, Table 1) whereas ketoconazole failed to induce ERMD activity (Table 1). The lack of detectable ERMD activity in microsomes isolated from ketoconazole-treated rats may be the result of low amounts of residual drug inhibiting P-450p as was observed with microsomes isolated 24 hours after the last clotrimazole treatment (see Appendix, figure 2).

Microsomal benzphetamine N-demethylase (BNZD) activity, a marker of phenobarbital-responsive cytochromes P-450b/e (Guengerich et al., 1982), was stimulated to the greatest extent by treatment with

phenobarbital (5.8-fold) or miconazole (5.1-fold, Table 1).

Clotrimazole also significantly stimulated BNZD activity (3.2-fold) whereas ketoconazole produced only a marginal increase (1.3-fold) in this activity (Table 1).

N-nitrosodimethylamine (NDMA) N-demethylase activity, a marker of the ethanol-inducible rat cytochrome P-450j (Yang et al., 1985; Ryan et al., 1986; Levin et al., 1986) gave more variable results, but was increased the greatest by miconazole (3 times the value in female controls, Table 1), slightly by ketoconazole and not significantly by clotrimazole (Table 1).

Identification and Quantitation of Immunoreactive Cytochromes

P-450- Representative qualitative immunoblots of liver microsomes from control and induced rats probed with antibodies raised against purified cytochromes P-450p, P-450b, and P-450j are shown in Figure 2. As reported previously, the anti-P-450p monoclonal antibody 1G8 reacts with P-450p and with a faster-migrating protein expressed in untreated male rats (see Chapter 1). The same bands are observed in microsomes from dexamethasone-treated female rats (Figure 3). The faster-migrating protein expressed in untreated male rats and in induced female rats represents a P-450p-related protein (see Chapter 1) which has not yet been isolated and characterized. The present studies revealed that imidazole drugs induce two 1G8-reactive proteins as does dexamethasone (Figure 3). The strong induction of the upper protein band precluded acceptable resolution and separate densitometric quantitation of the two immunoreactive proteins. The two bands measured as total anti-P-450p-reactive microsomal protein were markedly increased (more than 380-fold over control values) by clotrimazole

(Table 2). Clotrimazole induced immunoreactive P-450p in excess of that induced by TAO (221-fold, Table 2). 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 2). The inductions of immunoreactive P-450p protein were accompanied by increases in ERMD activity except for microsomes from ketoconazole-treated rats in which ERMD activity remained unchanged from control values (Tables 1 and 2).

The same liver microsomal samples were quantitatively analysed on immunoblots developed with anti-P-450b or anti-P-450j antibodies (Table 2). Miconazole and clotrimazole induced immunoreactive P-450b/e by 86- and 102-fold, respectively, whereas ketoconazole did not induce P-450b/e protein (Figure 2, Table 2). In contrast, among all the agents tested, only ketoconazole increased (2-fold) the level of immunodetectable P-450j (Table 2). NDMA N-demethylase activity was increased to a similar extent in the ketoconazole microsomes (Tables 1 and 2). In contrast, miconazole treatment increased NDMA N-demethylase activity 3-fold even though immunoreactive P-450j protein was not elevated (Table 2).

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 three P-450 cDNA probes was employed (Figure 4). The results of the quantitation studies, summarized in Table 3, showed that clotrimazole treatment increased hybridizable P-450p mRNA 93-fold over the control value, a rise much less than the corresponding 382-fold increase in immunoreactive P-450p protein (Tables 2 and 3). Similarly, TAO

produced a disproportionate increase in immunoreactive P-450p protein (221-fold) as compared to hybridizable P-450p mRNA (45-fold, Tables 2 and 3). In contrast, miconazole, ketoconazole, dexamethasone and phenobarbital each produced nearly identical increases over control values in P-450p immunoreactive protein and in hybridizable mRNA (Tables 2 and 3).

Less than proportionate increases in hybridizable P-450b/e mRNA as compared to total immunoreactive P-450b/e protein were also apparent following clotrimazole, miconazole, phenobarbital, or dexamethasone treatments (Tables 2 and 3). As expected, ketoconazole, which failed to increase either BNZD activity or immunoreactive P-450b/e protein, produced no change in hybridizable P-450b/e mRNA (Table 3).

None of the agents tested increased hybridizable P-450j mRNA including ketoconazole, the only imidazole which induced immunoreactive P-450j protein (Table 3). The latter observation is consistent with reports that treatment of rats with pyrazole, acetone, or 4-methylpyrazole increases immunodetectable P-450j protein and NDMA N-demethylase activity but does not increase P-450j mRNA (Wrighton et al., 1986; Song et al., 1986).

DISCUSSION

The results of this study demonstrate that N-substituted imidazoles including clotrimazole, miconazole and ketoconazole, represent a new class of inducers of the glucocorticoid-responsive family (Nebert et al., 1987) of hepatic cytochromes P-450. These antifungal drugs increased ERMD, an activity linked to P-450p, induced one or more immunoreactive P-450p proteins, and increased the amount of liver mRNA hybridizing to a P-450p cDNA probe. Indeed, clotrimazole now supplants TAO as the most effective known inducer of total CO-binding heme protein and of P-450p (Wrighton et al., 1985b; see also Chapter 1). Exact identification of the subtypes of P-450p-related protein(s) induced by these antifungal drugs will require further study.

The cytochrome P-450 inducing properties of the antimycotic drugs are not limited to members of the P-450p family. Catalytic, immunochemical and cDNA hybridization studies demonstrated that clotrimazole and miconazole, but not ketoconazole, are also effective inducers of the phenobarbital-inducible cytochromes P-450b/e. Indeed, at the doses administered, both miconazole and clotrimazole proved to be as effective as was phenobarbital in increasing total P-450b/e immunoreactive protein and hybridizable P-450b/e mRNA (Tables 2 and 3). Clotrimazole and miconazole can be grouped along with compounds such as chlordane, transnonaflor, halogenated biphenyls, and phenobarbital as shared inducers of the steroid- and phenobarbital-responsive cytochromes P-450 and provide further evidence that, in rat liver,

these two distinct families exhibit overlapping regulatory characteristics. This is in contrast to other families of cytochrome P-450, such as the aromatic hydrocarbon-inducible isozymes which exhibit stricter structure-activity requirements for inducibility and less overlap with inducers of other families of cytochrome P-450.

Several years ago, this laboratory investigated the mechanism underlying the dramatic induction of cytochrome P-450p by the macrolide antibiotic, TAO and found that in both hepatocyte cultures and in living rats, this drug selectively prolongs the half-life of P-450p (Watkins et al., 1986). Thus, TAO treatment results in a marked accumulation of P-450p protein while P-450p mRNA is increased only slightly (Wrighton et al., 1985b; Watkins et al., 1986). 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 (Tables 2 and 3). Thus, it seems likely that clotrimazole, like TAO, inhibits the rate of 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 (Wrighton et al., 1985; Pessayre et al., 1981). However, clotrimazole does not appear to form such a spectrally detectable complex with P-450p (Pershing et al., 1986). In this regard, clotrimazole resembles other types of P-450p inducers such as clordane or trans-nonaclor 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 (Schuetz et al., 1986). In contrast to clotrimazole, miconazole and ketoconazole increased P-450p protein and mRNA to similar extents as

did dexamethasone and phenobarbital (Tables 2 and 3). These findings suggest that the imidazole antifungal drugs may be useful probes for further study of the control of P-450p turnover.

A notable discrepancy became apparent when the magnitude of induction of P-450p mRNA and protein by dexamethasone observed in this study was compared with that reported earlier by Simmons and coworkers. These investigators found that a single dexamethasone injection increased hybridization of cytoplasmic poly(A) mRNA to a P-450PCN cDNA probe a maximum of 18-fold over control values, whereas chronic dexamethasone administration (80mg/kg daily for 4 days) elevated immunodetectable P-450PCN protein only 4-fold (Simmons *et al.*, 1987). In contrast, in the present study, dexamethasone was found to induce P-450p mRNA and protein 141-fold and 124-fold, respectively (Tables 2 and 3). 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.

As was the case with cytochrome P-450p, measurements of P-450b/e protein and mRNA following treatments with several inducers also revealed accumulation of immunoreactive protein in excess of hybridizable mRNA (Tables 2 and 3). In this regard, the imidazoles miconazole and clotrimazole produced the greatest discrepancy, elevating P-450b/e protein 5.4 and 3.6 times more than hybridizable P-450b/e mRNA, respectively, as derived from data presented in Tables 2 and 3. Less dramatic differences in accumulation of P-450b/e protein and mRNA resulted from phenobarbital or dexamethasone treatments (a ratio of increase in P-450b/e protein to mRNA of 2.3 and 2.2,

respectively). It is noteworthy that Simmons and coworkers found a 10- to 12-fold increase in hybridizable P-450b/e mRNA following dexamethasone treatment, nearly identical to the increase observed in the present study (Table 3, Simmons et al., 1987). Moreover, in this same report, these investigators suggested the possible existence of a steroid-inducible P-450b-related protein (or proteins) that have not yet been identified (Simmons et al., 1987). Indeed, in the present studies, it was observed that dexamethasone induced a qualitatively distinct pattern of microsomal proteins recognized by the anti-P-450b antibody. As shown in Figure 2, the major anti-P-450b-reactive band in microsomes isolated from dexamethasone-treated rats exhibits a slightly greater apparent molecular weight than the major protein band induced by phenobarbital, miconazole, or clotrimazole. Thus, the value reported for total immunoreactive P-450b/e in microsomes from dexamethasone-treated rats reflects the presence of not only cytochromes P-450b and P-450e, but other immunochemically related proteins.

The expression of cytochrome P-450j, another isozyme representing yet another cytochrome P-450 family, was stimulated by ketoconazole, but not by either clotrimazole or miconazole. Induction of P-450j by ketoconazole was evidenced by increases in both NDMA N-demethylase activity and immunodetectable P-450j, whereas levels of hybridizable P-450j mRNA were unchanged by ketoconazole treatment (Tables 1, 2 and 3). Thus, ketoconazole, like other inducers of P-450j, appears to affect the expression of this isozyme by post-translational mechanisms (Wrighton et al., 1986; Song et al., 1986). Moreover, it can be reasonably concluded that, as a class of P-450 inducers in the rat, the

N-substituted imidazoles are apparently capable of regulating at least three families of cytochrome P-450 isozymes by multiple mechanisms, one of which involves altered protein turnover.

In summary, the present findings add to the growing body of evidence that N-substituted imidazoles represent a unique and important class of agents which have multiple effects on the expression of rat hepatic cytochromes P-450. These drugs will undoubtedly serve as useful as tools in the ongoing investigation of the molecular mechanisms involved in the regulation of the hepatic cytochromes P-450. Obviously, the doses of clotrimazole, miconazole and ketoconazole administered 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 (Stricker et al., 1986). Recently, in addition to its use as an antifungal agent, this drug has been utilized as an inhibitor of steroid synthesis (Sonino, 1987) further emphasizing the importance of identifying the mechanisms by which ketoconazole and structurally similar compounds modulate the expression and function of cytochromes P-450. Because clotrimazole and miconazole are primarily used topically thereby limiting systemic absorption, 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 man.

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TABLE 1

EFFECT OF TREATMENTS ON MICROSOMAL N-DEMETHYLASE ACTIVITIES
(nmol/min/mg)

Treatment	Erythromycin	(X- control)	Benzphetamine	(X- control)	N-nitroso- dimethylamine	(X- control)
vehicle (F)	1.32 ± 0.09		1.24 ± 0.29		0.17 ± 0.04	
vehicle (M)	1.29 ± 0.20	(1.0)	2.46 ± 0.25 ^a	(2.0)	0.35 ± 0.04 ^a	(2.1)
Clotrimazole	9.61 ± 1.10 ^a	(7.3)	4.02 ± 0.54 ^a	(3.2)	0.35 ± 0.18	(2.1)
Miconazole	2.84 ± 0.38 ^a	(2.2)	6.35 ± 0.80 ^a	(5.1)	0.51 ± 0.11	(3.0)
Ketoconazole	1.26 ± 0.45	(1.0)	1.67 ± 0.22 ^b	(1.3)	0.32 ± 0.12 ^b	(1.9)
β-naphthoflavone	0.95 ± 0.22 ^a	(0.7)	1.09 ± 0.09	(0.9)	0.33 ± 0.04 ^a	(1.9)
Dexamethasone	8.60 ± 0.72 ^a	(6.5)	3.86 ± 0.45 ^a	(3.1)	0.25 ± 0.14	(1.5)
Phenobarbital	2.14 ± 0.34 ^a	(1.6)	7.18 ± 0.56 ^a	(5.8)	0.17 ± 0.05	(1.0)
TAO	10.97 ± 1.39 ^a	(8.3)	2.15 ± 0.11 ^a	(1.7)	0.39 ± 0.13 ^a	(2.3)

Microsomes were isolated from control or treated rats and erythromycin, benzphetamine, and N-nitrosodimethylamine N-demethylase activities were measured in vitro as described in Methods. For these studies, clotrimazole (100 mg/kg) was given daily for three days and microsomes were isolated 42 hours after the last treatment. Microsomes from TAO-treated rats were decomplexed with K_3FeCN_6 prior to incubation with substrate. Shown are the mean ± standard deviation of values for five individual rats.

^a $p < 0.01$

^b $p < 0.05$

TABLE 2

QUANTITATION OF IMMUNOREACTIVE CYTOCHROMES P-450 IN
LIVER MICROSOMES FROM RATS TREATED WITH INDUCERS

<u>Treatment</u>	<u>Anti-P-450p</u>	<u>Anti-P-450 b/e</u>	<u>Anti-P-450j</u>
vehicle (F)	0.008 ± 0.001 (1)	0.006 ^a	0.045 ± 0.016 (1)
vehicle (M)	0.013 ± 0.002 (1.6)	0.006 ^a	0.034 ± 0.004 (0.76)
Clotrimazole	3.054 ± 0.423 (382)	0.514 ± 0.190 (86)	0.031 ± 0.004 (0.69)
Miconazole	0.414 ± 0.116 (52)	0.613 ± 0.125 (102)	0.039 _b ± 0.013 (0.87)
Ketocanazole	0.429 ± 0.150 (54)	0.007 ± 0.002 (1)	0.095 ^b ± 0.011 (2.10)
β-naphthoflavone	0.008 ± 0.002 (1)	0.006 ^a	0.029 ^a
Dexamethasone	0.988 ± 0.318 (124)	0.138 ± 0.068 (23)	0.035 ± 0.011 (0.78)
Phenobarbital	0.211 ± 0.049 (26)	0.476 ± 0.132 (79)	0.040 ± 0.011 (0.89)
TAO	1.764 ± 0.447 (221)	0.019 ± 0.013 (3)	0.029 ^a

Immunoblot analyses of microsomes isolated from rats treated with vehicle or inducers (see figure 1) were performed as previously described with the purified cytochromes P-450 included as standards. The stained protein bands were quantitated with a Zeiss densitometer. The results are expressed as total immunoreactive protein, in nmol/mg, detected by each antibody along with the value relative to vehicle-treated female rats.

^a Value at or below lowest amount detectable

^b $p < 0.01$ compared with vehicle-treated female

TABLE 3
 QUANTITATIVE ANALYSES OF HYBRIDIZABLE P-450 mRNA

Treatment	P-450p		P-450b/e		P-450j	
	\bar{X}	control \pm SD	\bar{X}	control \pm SD	\bar{X}	control \pm SD
vehicle (M)	1.9	\pm 0.1	0.9	\pm 0.2	0.9	\pm 0.2
Clotrimazole	92.9	\pm 1.2	23.9	\pm 0.9	0.7	\pm 0.1
Miconazole	31.9	\pm 1.9	18.8	\pm 1.9	1.0	\pm 0.3
Ketoconazole	58.2	\pm 13.0	0.9	\pm 0.3	0.7	\pm 0.2
β -naphthoflavone	1.6	\pm 0.1	0.5	\pm 0.1	0.5	\pm 0.1
Dexamethasone	140.8	\pm 17.8	10.1	\pm 3.5	0.7	\pm 0.1
Phenobarbital	28.1	\pm 3.4	36.0	\pm 1.9	1.0	\pm 0.2
TAO	44.8	\pm 3.6	3.7	\pm 0.6	0.7	\pm 0.1

Identical samples of total liver RNA analyzed in Figure 4 were subjected to slot blot analyses as described in Experimental Procedures. RNA samples from each rat (0.05 to 30 μ g) were applied to nitrocellulose filters, hybridized with the indicated 32 P-labelled cDNA probe, visualized by autoradiography, and quantitated by scanning densitometry. The results are expressed as hybridization relative to RNA isolated from vehicle-treated female rats included on the same filter and represent the mean \pm the standard deviation of four to five individual rats.

Figure 1. Total Spectral P-450 in Liver Microsomes. Rats were treated with inducers (BNF, β -naphthoflavone; DEX, dexamethasone; PB, phenobarbital; TAO, triacetylolandomycin; CTZ, clotrimazole; MCZ, miconazole; KCZ, ketoconazole) or vehicle (F, female; M, male) as described in Methods. 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 maximum at 450nm reach a maximum. In the case of microsomes from TAO-treated rats, $K_3Fe(CN)_6$ was added to a final concentration of 20 μM three minutes prior to carbon monoxide. Shown are the mean \pm the standard deviation of values from five individual rats.

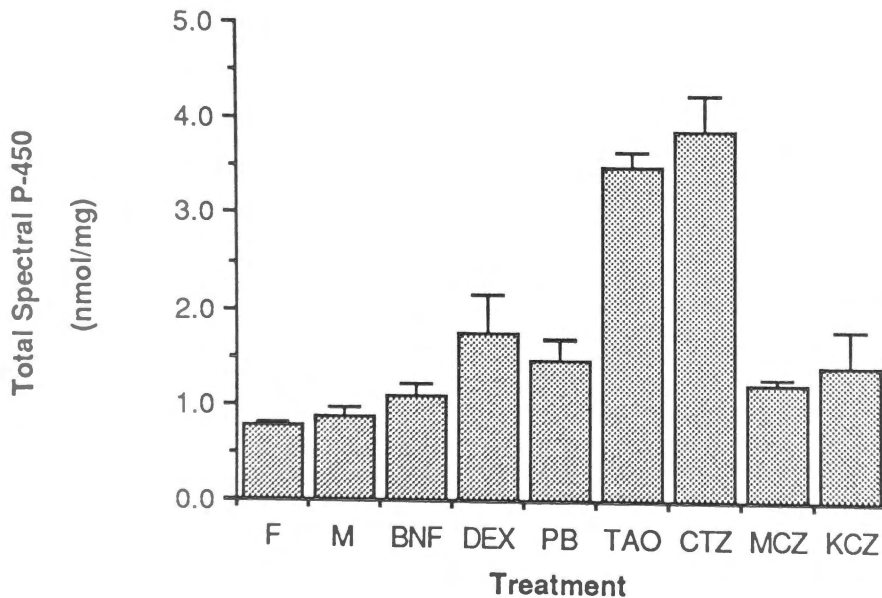


Figure 2. Immunoblot Analysis of Liver Microsomes Developed with Anti-P-450p. Microsomal samples were resolved electrophoretically in 10% SDS-polyacrylamide slab gels and then transferred to nitrocellulose filters which were subsequently incubated with anti-P-450p antibody. Immunoreactive protein bands were visualized with diaminobenzidine. Shown are microsomal samples from a vehicle-treated male rat (M, 50 μ g protein) and microsomes isolated from female rats treated with either dexamethasone (DEX, 3 μ g protein), clotrimazole (CTZ, 1.5 μ g protein), miconazole (MCZ, 15 μ g protein) or ketoconazole (KCZ, 10 μ g protein). Also shown is 1.0 pmole of purified P-450p.

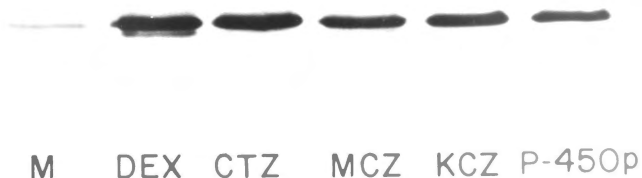


Figure 3. Immunoblot Analyses of Liver Microsomes. Microsomal samples from control or induced rats containing the indicated protein quantities were electrophoresed, blotted, and then developed with the indicated antibodies as described in Methods. Also included are purified cytochromes P-450p (top panel), P-450b (middle panel) and P-450j (bottom panel).

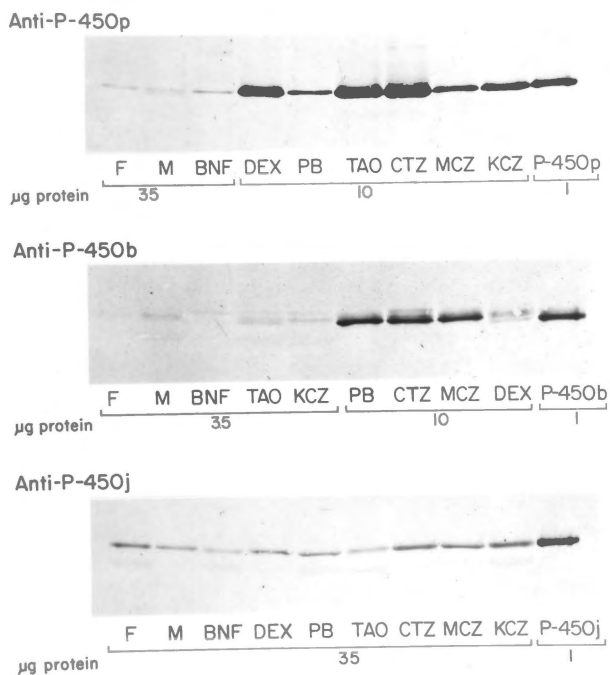
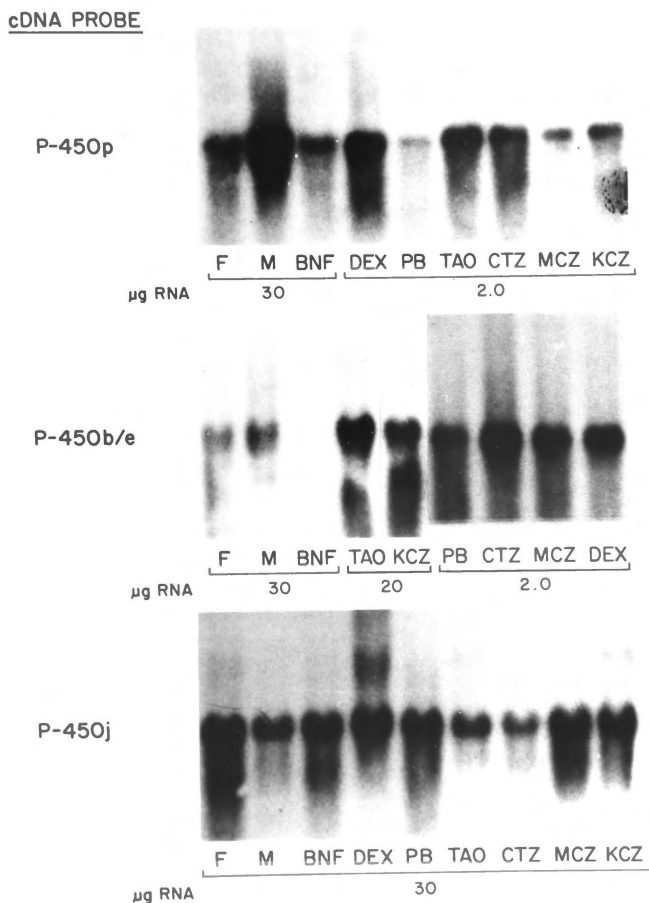


Figure 4. Northern Blot Analyses of Hybridizable P-450 mRNA. Total liver RNA isolated from rats treated as outlined in figure 1 was subjected to electrophoresis in agarose gels, transferred to nitrocellulose filters and then hybridized with the indicated 32 P-labelled cDNA probe as described in Methods. Hybridizable P-450 mRNA was visualized by autoradiography.



DISCUSSION

Since the isolation and purification of the major rat liver cytochrome induced by PCN reported by this laboratory (Elshourbagy & Guzelian, 1980), there has been extensive effort here and elsewhere to further characterize this protein and the mechanisms involved in its regulation. Strong, albeit indirect evidence suggesting the existence of a "PCN family" of cytochromes in the rat, analogous to other families of inducible P-450 isozymes, prompted the development of a battery of immunochemical probes to carry out more definitive investigations. The results from these studies further establish that the P-450p family is unique among the families of rat liver cytochromes P-450 identified.

On the basis of immunochemical evidence generated with highly specific monoclonal antibodies, it was concluded that at least three (and possibly four) P-450p-related forms are expressed in the liver of untreated rats. Both male-specific and female-specific members exist among these "control" forms of the P-450p family, an unusual feature among inducible cytochromes P-450 (Waxman, 1986). Moreover, expression of subtypes of P-450p-related proteins is differentially affected by xenobiotic inducers. In contrast, other inducible families of P-450, such as cytochromes P-450b and P-450e and cytochromes P-450c and P-450d rise in parallel in response to phenobarbital and aromatic hydrocarbons, respectively (Adesnick & Atchison, 1985; Whitlock, 1986).

Another striking feature of the P-450p family is the lack of structural similarity among chemical inducers that have been

identified. These include steroids, barbiturates, halogenated biphenyls, organochlorine pesticides, macrolide antibiotics, and most recently, imidazole antimycotic agents. From this observation, it is clear that the regulation of this family of isozymes is complex. Indeed, multiple mechanisms have been implicated in the induction of the major form, P-450p. Hormonal regulation accounts for the male-specific expression of P-450p in untreated rats (Waxman et al., 1985). Induction of P-450p by steroids clearly involves factors dissociated from the glucocorticoid receptor and possibly involves a unique "PCN receptor" (Scheutz & Guzelian, 1984). It is known that PCN stimulates the rate of transcription of the gene encoding P-450PCN (Hardwick et al., 1983) presumably the same protein as P-450p (Hostetler et al., 1987). It is noteworthy that dexamethasone, a strong inducer of both subtypes of the P-450p family, induces other P-450 isozymes, most notably P-450b/e, by prolonging the half-life or "stabilizing" messenger RNA (Simmons et al., 1987). Thus, it is possible that a single agent, dexamethasone, may regulate P-450p expression via multiple mechanisms.

Another previously identified mode of induction of P-450p involves the selective prolongation of the half-life of this isozyme which occurs following administration of triacetyloleandomycin. The result is a profound accumulation of P-450 protein, far in excess of the corresponding increase in P-450p mRNA (Watkins et al. 1986). In the present studies, a newly identified inducer of P-450p was found to markedly induce P-450p to a similar or even greater extent than does triacetyloleandomycin. Moreover, the disproportionate accumulation of P-450p protein relative to mRNA following clotrimazole treatment

strongly suggests that this drug also significantly retards the rate of P-450p catabolism. It is noteworthy that neither of the structural analogs miconazole nor ketoconazole share this apparent stabilizing effect on P-450p. Nevertheless, both clotrimazole and miconazole induce P-450b/e protein in excess of the corresponding mRNAs, and likewise, ketoconazole increases P-450j protein but causes no increase in the level of P-450j mRNA. These observations by no means prove, but are consistent with the testable hypothesis that these imidazole antifungal drugs regulate multiple P-450 isozymes in a selective fashion by altering rates of hemeprotein turnover.

Recent observations in this laboratory suggest that the P-450p homologue in man, cytochrome H1p, is also a member of a multigene family. Thus, glucocorticoid-responsive cytochrome(s) P-450 in human liver not only serve as a useful model for further studies of the mechanisms of eucaryotic gene regulation, but also represent an important enzyme system prone to modulation by commonly used drugs including glucocorticoids, barbiturates, macrolide antibiotics, and imidazole antimycotic agents. Continued investigation in this area will undoubtedly enhance basic understanding of this important enzyme system as well as provide a basis for rational and effective drug therapy.

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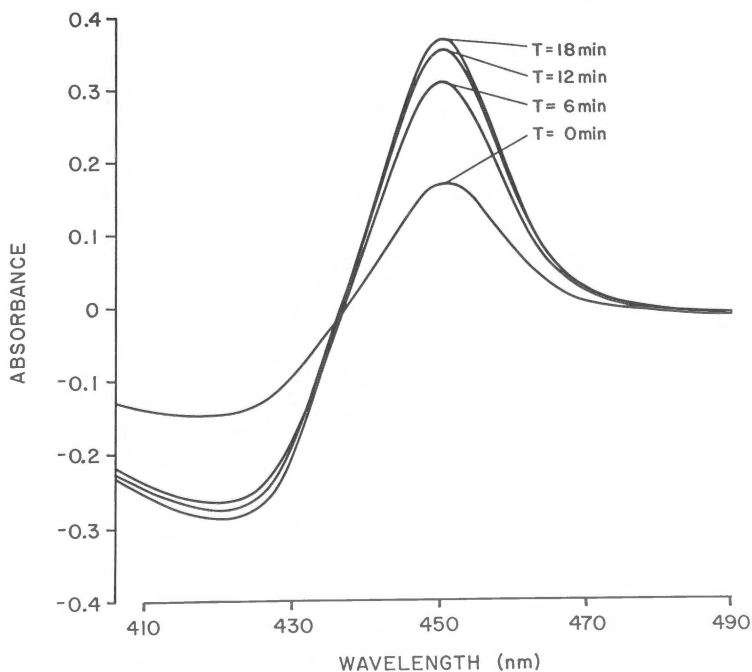
APPENDIX

Appendix Figure 1. Low level expression of P-450p in untreated female rats. Shown is an immunoblot of 50 μ g of microsomal protein from an untreated female rat (F) and 4.0 μ g from a clotrimazole-treated female rat (CTZ) developed with anti-P-450p monoclonal antibody 1G8. As described in Chapter 1, the detection of immunoreactive P-450p in microsomes from untreated female rats requires the analysis of quantities of protein which exceed the limit established for quantitative electrophoretic transfer, which is 35 μ g.



F CTZ

Appendix Figure 2. Time course for saturation of CO binding to cytochrome P-450 in liver microsomes isolated from rats treated with clotrimazole at a dose of 150mg/kg daily for 3 days. Microsomes were isolated 24 hours after the last clotrimazole treatment. Microsomal suspensions were diluted to 1.0mg/ml, reduced with sodium dithionite, and then split between sample and reference cuvettes of an Aminco DW-2a dual beam spectrophotometer. After a baseline recording, CO was bubbled through the sample cuvette for 60 seconds and then successive difference spectra between 360 and 510nm were recorded at the indicated time points. As described in Chapter 2, the slowly developing CO-difference spectrum is indicative of competition between CO and a compound tightly bound to heme.



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