ABSTRACT:

Cytochrome P-450 (CYP) 2J4 is a member of the recently identified CYP2J subfamily—part of the CYP superfamily—and is primarily expressed in rat small intestinal epithelium (enterocytes). Studies to determine small intestinal CYP2J4 inducibility by prototypic CYP inducers have been undertaken. Immunoblot analysis of enterocyte microsomes from rats treated with β-naphthoflavone, dexmethasone, or phenobarbital revealed unchanged, diminished, or slightly increased levels of CYP2J4 protein, respectively, relative to vehicle-treated rats, whereas rats treated with pyrazole (200 mg/kg) had 3- to 4-fold increased levels of CYP2J4. Pyrazole administration also increased CYP2J4 metabolic activity, as probed by retinoic acid formation from retinal, approximately 3-fold, and the activity was inhibited by 90% by a polyclonal anti-CYP2J4 antibody. CYP2J4 mRNA levels were increased 2.5-fold by pyrazole administration. The route of pyrazole administration—oral or i.p.—did not affect the extent or time course of intestinal CYP2J4 induction. However, at >300 mg/kg pyrazole, oral administration produced higher levels of CYP2J4 activity than i.p. administration. Pyrazole also produced increased hepatic and olfactory mucosal levels of CYP2J4. We speculate, based on our data and on published mechanisms of pyrazole induction, that pyrazole induces rat intestinal CYP2J4 by stabilization of mRNA primarily, and by stabilization of protein to a lesser extent. This study documents for the first time the induction of a CYP2J subfamily member by a xenobiotic and provides the basis for a mechanism by which xenobiotics could modulate biological processes.

Several members of the cytochrome P-450 (CYP) 2J subfamily of the CYP superfamily have been identified since the first report on the expression of CYP2J1 in rabbits (Kikuta et al., 1991). These include CYP2J2 in humans (Wu et al., 1996), CYP2J3 in rats (Wu et al., 1997), CYP2J4 in rats (Zhang et al., 1997), CYP2J5 and 2J6 in mice (Ma et al., 1998), and CYP2J9 in mice (Qu and Zeldin, 1999). These CYPs exhibit selective tissue expression with CYP2J1 mRNA specifically expressed in the small intestine (Kikuta et al., 1991). CYP2J2 mRNA is expressed primarily in the heart with more limited expression in the liver and small intestine and CYP2J2 protein is similarly expressed (Wu et al., 1996). CYP2J3 mRNA is expressed primarily in the liver and to much lesser extents in the heart, lung, kidney, stomach, and small intestine; CYP2J3 protein is expressed primarily in the liver and heart, and to a lesser extent in the lung, kidney, stomach, small intestine, and colon (Wu et al., 1997). CYP2J4 mRNA and protein are expressed predominantly in the small intestine and to a lesser extent in liver and olfactory mucosa (Zhang et al., 1997). CYP2J5 mRNA and protein are expressed primarily in kidney and liver (Scarborough et al., 1999). CYP2J6 mRNA is expressed in intestine with lower levels of expression in heart, lung, brain, kidney, and liver (Scarborough et al., 1999). CYP2J9 mRNA is expressed primarily in brain and kidney with less expression in liver, intestine, heart, lung, and ovary; CYP2J9 protein is expressed in brain and kidney (Qu and Zeldin, 1999).

The varying but overlapping selectivities of organ expression of this range of CYP2J forms is provocative. There are, however, limited data available on the functions of the CYP2J subfamily. Several substrates have been identified, including arachidonic acid, which is metabolized to epoxyeicosatrienoic acids, hydroxyeicosatetraenoic acids, and ω-1-alkols of arachidonic acid by CYP2J2, 2J3, 2J4, and 2J5 (Wu et al., 1996, 1997; Zhang et al., 1997; Scarborough et al., 1999); benzoatetramine, which is metabolized by CYP2J1, 2J3, and at a much lower rate by 2J2 (Kikuta et al., 1991; Wu et al., 1996, 1997); and all-trans- and 9-cis-retinal, which are metabolized to the corresponding retinoic acids (RAs) by CYP2J4 (Zhang et al., 1998). Several other substrates, which have low turnover rates catalyzed by CYP2J2s, have been identified. These include testosterone catalyzed by CYP2J2, 2J3, and 2J5; diclofenac and bufuralol catalyzed by CYP2J2 and 2J5; and progesterone and warfarin catalyzed by CYP2J2 (Zhang et al., 1997; Scarborough et al., 1999). The CYP2J2-mediated metabolism of retinoids and arachidonic acid could implicate these CYPs in regulation of the function of organs in which they are expressed.

The most glaring deficiency in our knowledge of the CYP2J subfamily is that of its regulation of expression. The few studies published have reported only negative results. This had led to the assumption that CYP2J2s are only constitutively expressed (Scarborough et al., 1999). Thus, known inducers of other forms of CYP did not affect levels of expression of CYP2J3 in rats as assessed by immunoblotting of any of the relevant organs (Zeldin et al., 1996, 1997a; Wu et al., 1997). Levels of hepatic CYP2J3 in rats were not altered by fasting and refeeding (Qu et al., 1998) or in rats with salt-sensitive hyperten-

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1 Abbreviations used are: CYP, cytochrome P-450; BNF, β-naphthoflavone; PB, phenobarbital; DEX, dexamethasone; TBST, 20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and 0.05% Tween-20; RA, retinoic acid.
section, cardiac ischemia/reperfusion, or oxygen-induced lung injury (Zeldin et al., 1997a).

In an attempt to determine whether any of the common inducers of CYPs could affect rat intestinal CYP2J4 regulation, we have investigated the effects of administered phenobarbital (PB), β-naphthoflavone (BNF), pyrazole, or dexamethasone (DEX) on CYP2J4 mRNA and protein levels and on activity toward retinal.

Materials and Methods

Treatment of Animals. Male Wistar rats (220–250 g b.wt.) were obtained from a colony maintained by the Wadsworth Center. Animals were kept at 22°C with a 12-h light/dark cycle and allowed free access to water and a pure diet (AIN-76A Diet; Dyets Inc., Bethlehem, PA). For induction studies, rats were treated by i.p. or oral administration of an inducing agent for indicated times and were sacrificed by CO2 overdose. The inducing agents and the doses were treated by i.p. or oral administration of an inducing agent for indicated diet (AIN-76A Diet; Dyets Inc., Bethlehem, PA). For induction studies, rats from a colony maintained by the Wadsworth Center. Animals were kept at

Isolation of Intestinal Epithelial Cells and Preparation of Microsomes. Small intestinal tissues from three rats were combined for each microsomal preparation. Intestinal epithelial cells were isolated by differential elution and microsomes were prepared from these cells as described previously (Pasco et al., 1993). Microsomes were stored at −80°C before use. Microsomal protein concentrations were determined using bicinchoninic acid reagent (Pierce Chemical Co., Rockford, IL) with BSA as the standard.

Immunoblot Analysis. Microsomal proteins were separated by SDS-polyacrylamide gel electrophoresis as described previously (Laemmli, 1970), in 10% polyacrylamide gels. Each sample was loaded at 10 µg protein/well. The resolved proteins were electrophoretically transferred to nitrocellulose sheets (Towbin et al., 1979), which were then treated with 5% nonfat dry milk in 20 mM Tris-HCl (pH 7.4) containing 0.5 M NaCl and 0.05% Tween-20 (TBST) for 1 h at room temperature, incubated with a polyclonal anti-CYP2J4 antibody (Zhang et al., 1998) or a monoclonal anti-CYP2E1 antibody (Ding et al., 1991) in TBST containing 2.5% milk for an additional hour, washed with TBST, and then incubated with a secondary antibody at 1:10,000 dilution in TBST containing 2.5% milk. The secondary antibody was a peroxidase-labeled goat antirabbit IgG or rabbit antimonine IgG (Sigma) and was detected with an enhanced chemiluminescence kit as described by the manufacturer (Amer sham, Arlington Heights, IL). In some experiments, the optical intensity of each immunoreactive band was determined by scanning with a Pharmacia-LKB densitometer.

Preparation and Analysis of RNA. Total RNA was prepared from small intestinal epithelial cells of male Wistar rats according to the method of Chomczynski (1993), with use of TRI Reagent obtained from the Molecular Research Center (Cincinnati, OH). Tissues from three rats were pooled for each preparation. RNA concentration and purity were determined spectrophotometrically, and the integrity of the RNA samples was assessed by ethidium bromide staining after agarose gel electrophoresis and by RNA blot analysis with a β-actin cDNA probe from Clontech (Palo Alto, CA). Poly(A) RNA was isolated from total RNA with use of an Oligotex mRNA isolation kit from Qiagen (Chatsworth, CA). RNA blot analysis was performed as described previously (Zhang et al., 1997). Hybridization was carried out for 2 h at 58°C in a Stratagene Quickhyb hybridization solution containing 32P-labeled cDNA probes. The probes were purified with a QIAquick kit from Qiagen after

Results

Metabolism of All-trans-retinal. Retinal metabolism was assayed essentially as described previously (Zhang et al., 1998). Reaction mixtures contained 50 mM potassium phosphate buffer, pH 7.4, 1 mM l-ascorbic acid, 30 µg of 1,2-dilauroyl-sn-glycero-3-phosphorylcholine, 0.5 mg microsomal protein and 100 µM all-trans-retinal. All mixtures were preincubated at 37°C for 1.0 min before the reaction was initiated with 20 µl of a 25 mM NADPH stock solution; control experiments were performed in which NADPH was omitted.

Reactions were carried out for 15 min at 37°C, during which the rates of product formation were linear with time. Reactions were terminated by quenching with 2.0 ml of ethyl acetate containing 50 µg/ml butylated hydroxytoluene. After the ethyl acetate extract was removed, the remaining aqueous solution was acidified with 10 µl of 88% formic acid and extracted with an additional 2.0 ml of ethyl acetate. The extracts were combined, the solvent was evaporated, and the residue was dissolved in 0.10 ml of methanol for analysis by HPLC as described in a previous study (Zhang et al., 1998). All reactions and other procedures were carried out in the absence of overhead light. RAs were quantified using the peak area at 360 nm, and all standard curves were linear over the concentration range of the RA products. The sources of retinoids, metabolite standards, and other reagents have been described previously (Zhang et al., 1998).

The inducibility of CYP2J4 in rat small intestine by compounds known to induce other forms of CYP was first examined by immunoblot analysis of intestinal microsomal protein. The protein was prepared from animals treated with the various inducers at dosages reported to affect induction of other CYPs. As shown in Fig. 1A, the levels of CYP2J4 protein were elevated in small intestine of pyrazole-treated rats and, to a lesser extent, in PB-treated rats relative to vehicle-treated controls. BNF treatment did not affect CYP2J4 protein level, whereas DEX treatment reduced the level of CYP2J4 protein. The induction of CYP2J4 in rat small intestine by pyrazole was
observed after either oral or i.p. administration of the inducing reagent, as shown in Fig. 1B. In both cases, levels of CYP2J4 protein were elevated 3- to 4-fold after treatment of rats with pyrazole. Our preliminary study demonstrated that treatment of rats for three consecutive days yields maximal induction.

Because pyrazole is a known CYP2E1 inducer (Wu and Cederbaum, 1994), immunoblot analyses using an anti-CYP2E1 antibody were also conducted to determine the inducibility of CYP2E1 in rat liver, kidney, and small intestine. Although CYP2E1 protein levels were elevated significantly in liver and kidney after pyrazole treatment, no CYP2E1 was detected in rat small intestine either before or after pyrazole treatment (data not shown), indicating that the protein detected in small intestine is not due to cross-reaction of the antibody with CYP2E1. The induction of CYP2J4 by pyrazole was further confirmed by RA biosynthetic activity measurements; CYP2J4 catalyzes intestinal microsomal metabolism of retinal to RA (Zhang et al., 1998). As shown in Fig. 2, the rate of RA formation was more than 3 times greater in small intestinal microsomes from pyrazole-treated rats relative to control rats. Almost 90% of RA microsomal synthesis activity was inhibited by the polyclonal anti-CYP2J4 antibody at 1 mg IgG/mg microsomal protein. Additional increases in IgG levels did not increase the extent of inhibition. These results support the conclusion that the induced activity was due to increased CYP2J4 expression.

To further characterize CYP2J4 induction by pyrazole in rat small intestine, the effects of pyrazole dosage administered orally or i.p. on this induction were examined. Rates of formation of RA catalyzed by intestinal microsomes of rats treated with different doses of pyrazole, either orally or i.p., are shown in Fig. 3A. With an increasing oral dose of pyrazole up to 300 mg/kg b.wt. per day for 3 days, there were incremental increases in retinal metabolism by intestinal microsomes in vitro. However, a further increase of the oral dose to 400 mg/kg led to a lower extent of induction, probably due to toxicity. When pyrazole was administrated i.p., maximal induction was observed at 200 mg/kg b.wt. and, similar to the situation with oral dosing, a lower extent of induction was observed when the dose was increased to 300 mg/kg.

The time course of CYP2J4 induction by pyrazole in rat intestinal epithelial cells was also investigated. The rates of formation of RA catalyzed by the intestinal microsomal preparations as a function of time after a single dose of pyrazole administered to the rats are shown in Fig. 3B. Small intestinal RA synthesis activity increased after pyrazole treatment either orally or i.p., reaching a maximum value at between 24 and 48 h and falling sharply thereafter.

To determine whether the induction of CYP2J4 occurs at the RNA level, RNA-blot analyses were undertaken with poly(A)$^+$ RNA from intestinal epithelial cells of control or pyrazole-treated rats, using a cDNA fragment from the coding region of CYP2J4 as a probe (Fig. 4). Qualitative analysis of the intensity of the CYP2J4 mRNA bands, corrected for by the amount of $\beta$-actin mRNA detected in each sample (Fig. 4B), indicated that the level of CYP2J4 mRNA was elevated about 2.5-fold in small intestine of pyrazole-treated rats compared with those from control animals, suggesting that a pretranslational
mechanism, at least in part, contributed to the increase of CYP2J4 protein.

The tissue-specificity of CYP2J4 induction by pyrazole was also examined. As shown in Fig. 5, the level of CYP2J4 protein was elevated in liver and olfactory mucosa, in addition to small intestine, suggesting that the induction is not small intestine-specific.

Discussion

The current study is the first to report induction of a member of the CYP2J subfamily. The physiological implications of this induction of CYP2J4 by pyrazole are, however, not clear because its functions have not been thoroughly elucidated. The capacity of CYP2J4, which is expressed in the small intestine to catalyze the metabolism of retinals to RA, and arachidonic acids to a variety of products (Zhang et al., 1997, 1998) suggests possible consequences of CYP2J4 induction. Thus others have speculated that intestinal CYP2J, through its catalysis of arachidonic acid metabolism, is involved in the release of neuropeptides, control of motility in the intestine, and modulation of fluid/electrolyte transport in the intestine (Zeldin et al., 1997), all of which could be affected by induction of CYP2J4. Increased CYP2J4 activity in the small intestine would also increase levels of RA, which could influence epithelial cell growth and differentiation (Duester, 1996). This possibility must be tempered by the fact that another, probably more predominant, pathway of RA synthesis involving intestinal aldehyde dehydrogenases is operative (Seitz and Oneta, 1998).

Our observations that pyrazole treatment of rats leads to concomitant increases in small intestinal CYP2J4 mRNA levels, protein levels, and RA formation activity is suggestive of a pretranslational mechanism for the induction. However, CYP2J4 protein levels were increased 3- to 4-fold, RA formation rates were increased just over 3-fold and CYP2J4 mRNA levels were increased approximately 2.5-fold. The slightly increased extent of CYP2J4 protein expression relative to increases in CYP2J4 mRNA expression after pyrazole administration suggests that CYP2J4 protein stabilization may also possibly be contributing to the overall induction of CYP2J4. Pyrazole has been previously reported to induce other CYP2 family members. In the case of CYP2E1, pyrazole induction occurs via stabilization of the protein (Winters and Cederbaum, 1992; Wu and Cederbaum, 1994). Pyrazole also induces CYP2A5 and, in mouse liver, the mechanism of this induction is mRNA stabilization (Aida and Negishi, 1991). Thus, based on our results and on reported mechanisms, the induction of CYP2J4 by pyrazole in rat small intestine is likely to occur via mRNA stabilization, with possibly a minor contribution by protein stabilization.

The current investigation of the role of the route of pyrazole administration on small intestinal CYP2J4 induction has revealed that oral and i.p. administration (200 mg/kg/day for 3 days) produced the same extent of induction of small intestinal CYP2J4 with same time course of induction (200 mg/kg for 1 day), whereas the dose responses were slightly different. Oral administration of high dosages of pyrazole (300–400 mg/kg) produced higher extents of enterocyte CYP2J4 activity than was the case with i.p. administered pyrazole. Oral administration of pyrazole will result in direct exposure of the enterocytes with a resultant initial high enterocyte concentration of pyrazole, in contrast with i.p. administration, which requires absorption in the peritoneum and systemic transportation to the enterocytes. Oral administration of pyrazole will result in direct exposure of the enterocytes with a resultant initial high enterocyte concentration of pyrazole, in contrast with i.p. administration, which requires absorption in the peritoneum and systemic transportation to the enterocytes. Oral administration of pyrazole would reasonably be expected to more rapidly and effectively induce intestinal CYP2J4 than i.p. administration, but this would only be expected to occur at time periods much shorter than our 24-h initial time point.

The short duration of induced CYP2J4 levels in the intestine–levels returned to control values after 3 to 4 days–is probably reflective of the short half-lives of rat enterocyte. These cells are sloughed off from
the intestinal villi approximately 2 days after beginning migration from the crypt surface (Iatropolous, 1986).

Although CYP2J4 is predominantly expressed in the small intestine of the rat, it is also expressed in the liver and olfactory mucosa (Zhang et al., 1997). The current studies determined that CYP2J4 levels were induced in all three organs, indicating a lack of tissue specificity in the inductive capacity of pyrazole.

Although CYP2E1 was eliminated as a possible confounder of our CYP2J4 induction data, the possibility of CYP2J3 influencing our conclusions must be considered. CYP2J3 is also expressed in rat small intestine (Wu et al., 1997; Zeldin et al., 1997), but at constitutive levels very much lower than those of CYP2J4 (Zhang et al., 1997). It is thus possible that, because the antibody used in our immunoblots cross-reacts with CYP2J3, some small contribution to the band corresponding to induced CYP2J4 arises from the CYP2J3.

In summary, we have demonstrated that CYP2J4 is induced in rat small intestine, as well as in liver and olfactory mucosa, by pyrazole. This induction is probably a consequence of mRNA and, less significantly, protein stabilization by pyrazole.

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