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ROLE OF INDUCTION OF SPECIFIC HEPATIC CYTOCHROME P450 ISOFORMS IN EPOXIDATION OF 4-VINYLCYCLOHEXENE

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ABSTRACT:

4-Vinyl-1-cyclohexene (VCH) is ovotoxic in B6C3F$_1$ mice but not in Fischer-344 rats, which can be partially attributed to greater formation of toxic epoxides from VCH in mice compared with rats. Since repeated exposure to VCH is necessary to cause ovotoxicity in mice, it is important to determine whether repeated exposure results in induction of cytochrome P450 (CYP) enzymes involved in its bioactivation. Hepatic microsomes prepared from mice or rats treated repeatedly with VCH demonstrated significantly increased VCH bioactivation in vitro, as assessed by VCH-1,2-epoxide, VCH-7,8-epoxide, or vinylcyclohexene diepoxide (VCD) formation. Mice and rats were then dosed with VCH, VCH-1,2-epoxide, or VCD for 10 days and measured for increases in hepatic microsomal CYP levels or activities. Total hepatic CYP levels were elevated only in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. Immunoblotting analysis of microsomes from VCH-treated rodents revealed elevated levels of CYP2A and CYP2B in mice but not rats. VCH-1,2-epoxide pretreatment also increased CYP2B levels in the mouse. Activities toward specific substrates for CYP2A and CYP2B (coumarin and pentoxyresorufin, respectively) confirmed that VCH and VCH-1,2-epoxide pretreatments resulted in increased catalytic activities of CYP2A and CYP2B in the mouse but not the rat. Pretreatment with phenobarbital, a known inducer of CYP2A and CYP2B, increased VCH bioactivation in both species. Interestingly, metabolism studies with human CYP "Supersomes" reveal that, of eight isoforms tested, only human CYP2E1 and CYP2B6 were capable of significantly catalyzing VCH epoxidation, whereas CYP2B6, CYP2A6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of the monoepoxides.

4-Vinyl-1-cyclohexene (VCH[1]) is formed by the spontaneous dimerization of two molecules of 1,3-butadiene during the rubber curing process (Rappaport et al., 1976; International Agency for Research on Cancer, 1994). VCH is also an intermediate in the synthesis of styrene and vinylcyclohexene diepoxide (VCD) for epoxy resin formation (International Agency for Research on Cancer, 1994). Repeated exposure of mice to VCH causes premature ovarian failure by depletion of ovarian primordial and primary follicles (Collins and Manus, 1987; Smith et al., 1990a; Hooser et al., 1994). This loss of follicles results in premature ovarian failure, which may be associated with the ovarian neoplasms that develop in mice chronically exposed to VCH (National Toxicology Program, 1986; Collins et al., 1987). Cytochrome P450 (CYP)-catalyzed bioactivation of VCH to epoxide metabolites (VCH-1,2-epoxide, VCH-7,8-epoxide, and ultimately, VCD) (Fig. 1) is necessary for this ovarian toxicity to occur (Smith et al., 1990b, Doerr et al., 1996). Interestingly, female rats are resistant to the ovarian toxicity caused by treatment with VCH, which is at least partially related to a reduced capacity to bioactivate VCH to the epoxide metabolites (Smith et al., 1990c). Understanding the molecular basis for this enhanced ability of the mouse to bioactivate VCH is necessary to better extrapolate which of these animal models, mouse or rat, would better predict the risk to humans exposed to VCH.

Studies in the mouse have established that repeated daily dosing with VCH is necessary for depletion of ovarian follicles (Smith et al., 1990b) and causes increases in total CYP protein and increases in VCH bioactivation in vitro (Doerr-Stevens et al., 1999). Furthermore, repeated exposure to VCH led to significantly elevated circulating levels of VCH-1,2-epoxide and VCD in the mouse in vivo. These results have prompted an investigation into the roles of specific CYP isoforms in hepatic VCH bioactivation in female B6C3F$_1$ mice and Fischer-344 rats. Through chemical inhibition and immunoinhibition techniques, it was demonstrated that CYP2A and CYP2B are involved in VCH bioactivation in the B6C3F$_1$ mouse (Smith et al., 1990c). Current studies focus on the roles of these and several other CYP subfamilies in species-dependent VCH bioactivation using techniques such as chemical induction, specific CYP activity studies and immunoblotting, and metabolism by different human heterologously expressed CYP proteins. In addition, mice and rats were dosed with VCH-1,2-epoxide or VCD (equitoxic doses to that of VCH in the mouse; Smith et al., 1990b) to determine whether VCH or the epoxide metabolites of VCH were causing the CYP induction.

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[1] Abbreviations used are: VCH, 4-vinyl-1-cyclohexene; VCD, vinylcyclohexene diepoxide; CYP, cytochrome P450; PB, phenobarbital.

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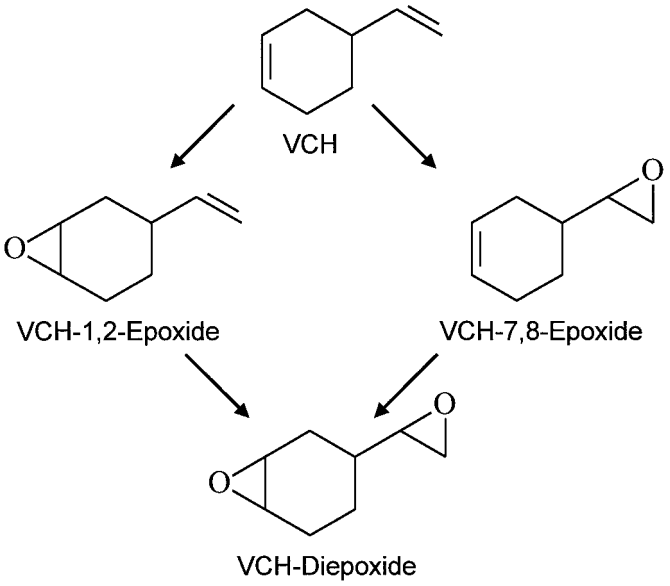

FIG. 1. *Proposed scheme for the hepatic bioactivation of VCH.*

Materials and Methods

Animals and Treatments. Female B6C3F$_1$ mice and Fischer-344 rats (approximately 28–38 days of age) were obtained from Harlan Bioproducts for Science (Indianapolis, IN). The animals were housed in cages with sawdust bedding and had free access to food (Harlan Teklad, Madison, WI) and water. Animals were maintained on a 12-h light/dark cycle and acclimated to this environment for at least 7 days before dosing and/or preparation of hepatic microsomes. Animals were dosed with either VCH (7.5 mmol/kg i.p. for 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), VCD (0.4 mmol/kg i.p. for 10 days) (Doerr-Stevens et al., 1999), or phenobarbital (PB) (80 mg/kg i.p. for 5 days). VCH-7,8-epoxide was not tested because of limited availability. PB was prepared as a 3.2% solution (w/v) in 0.9% NaCl. VCH, VCH-1,2-epoxide, and VCD were prepared in a sesame oil vehicle. In each treatment group, microsomes were prepared from four individual rats or were pooled from four mice per group (16 mice total).

Chemicals. VCH, VCH-1,2-epoxide, VCD, and methylcyclohexene were purchased from Aldrich (Milwaukee, WI). $NADP^+$, G6PDH, G6P, NADPH, trichloroacetic acid, coumarin, 7-hydroxycoumarin, pentoxyresorufin, ethoxyresorufin, resorufin, *p*-nitrophenol, *p*-nitrocatechol, and sodium borate were purchased from Sigma (St. Louis, MO). Sodium hydroxide was purchased from Fisher Scientific (Pittsburgh, PA). Magnesium chloride, phenobarbital sodium, and perchloric acid were purchased from Mallinckrodt (St. Louis, MO). Chloroform was purchased from Burdick and Jackson Products (Muskegon, MI). Cyclohexene oxide was a gift from NTP/RTI (Research Triangle Park, NC). VCH-7,8-epoxide was synthesized by the method of Watabe et al. (1981). For immunoblotting studies, anti-human polyclonal CYP2E1 primary antibody and anti-rat polyclonal CYP1A1, CYP2B1, CYP2C6, CYP3A2, and CYP4A1 primary antibodies were purchased from GENTEST (Woburn, MA), whereas the corresponding anti-goat secondary antibodies were purchased from Sigma. Anti-rat polyclonal CYP2A6 antibody was purchased from Affinity Bioreagents (Golden, CO), and anti-rabbit secondary antibody was purchased from Sigma.

Subcellular Preparations and Characterization. Animals were killed by inhalation of carbon dioxide 24 h after final dosing. Livers were excised and homogenized in a 50 mM Tris-HCl buffer (pH 7.4) using a drill motor and Teflon-glass homogenizer. Microsomes were prepared by subjecting this homogenate to differential centrifugation, as described by Guengerich (1989). Protein concentrations were determined by using a bicinchoninic acid kit (Pierce, Rockford, IL). Total P450 concentration (nmol/mg of microsomal protein) was determined using the carbon monoxide-binding spectrophotometric assay, as described by Omura and Sato (1964).

Capillary Gas Chromatographic Conditions for Epoxide Analysis. Analyses were performed on a Hewlett-Packard HP 5890A gas chromatograph equipped with a 0.25-mm diameter DB-624 capillary column (J&W Scientific, Inc., Folsom, CA) and a flame ionization detector. The nitrogen carrier gas flow rate was 1 ml/min. The flame ionization detector gas flow rates for H_2, N_2, and air were 42, 35, and 400 ml/min, respectively. Splitless injection was used with the purge-off from time 0 to 1.0 min with a 2-μl injection volume. The injection and detector temperatures were held isothermally at 200 and 250°C, respectively. The oven temperature was held at 60°C for 10 min, then increased to 230°C at a rate of 12°C/min. Final temperature was held for 3 min to ensure elution of the diepoxide. Retention times were 11.9 min for methylcyclohexene, 15.2 min for cyclohexene oxide, 16.6 min for VCH, 20.4 min for VCH-1,2-epoxide, 21.4 min for VCH-7,8-epoxide, and 25.0 min for VCD. VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD formation (nmol/mg of microsomal protein) was quantified by comparing the peak areas with those in standard curves of known amounts of the epoxides.

In Vitro Metabolism of VCH, VCH-1,2-Epoxide, and VCH-7,8-Epoxide in the Mouse and Rat. Microsomal protein (1 mg/ml) was added to a 50 mM HEPES/0.1 mM EDTA buffer containing a recycling NADPH system (0.5 mM $NADP^+$, 1 unit/ml G6PDH, and 10 mM G6P), 2 mM cyclohexene oxide (an epoxide hydrolase inhibitor; Guest and Dent, 1980), and 1 mM VCH. Previous studies demonstrated that these reactions are linear up to 1 mg/ml protein (data not shown). After 60 min, the cytochrome P450 reactions were terminated by submersion in liquid nitrogen. VCH and its epoxide metabolites (VCH-1,2-epoxide, VCH-7,8-epoxide, and VCD) were extracted with ethyl acetate containing 1 μl/ml methylcyclohexene as an internal standard. The epoxide metabolites were identified and quantified using gas chromatography, as described above. Data are presented as nanomole per milligram of microsomal protein formed. The values were adjusted for percent recovery of epoxides, which were approximately 96% for VCH-1,2-epoxide, 92% for VCH-7,8-epoxide, and 76% for VCD. The percentages of recoveries were calculated by injecting a known amount of compound into denatured microsomes (first heated for 30 min at 60°C) and then measured by gas chromatography.

Analysis of Specific CYP Levels in Microsomal Samples from Mice or Rats Pretreated with VCH. The immunoblot assay was performed under linear conditions. Depending on the relative hepatic expression of the CYP isoform to be assayed, either 1 or 10 μg of hepatic microsomal protein from each treatment group was used per gel lane. Proteins were separated on 10% SDS-polyacrylamide gel electrophoresis gels and transferred to nitrocellulose. Blots were incubated with polyclonal antibody to human CYP2E1 or polyclonal antibody to rat CYP1A1, CYP2B1, CYP2A6, CYP2C6, CYP3A2, or CYP4A11 for 1 h at room temperature. Blots were then incubated with anti-goat secondary antibody (for CYP1A1, CYP2B1, CYP2C6, CYP2E1, CYP3A2, or CYP4A11) or anti-rabbit secondary antibody (CYP2A6), all conjugated with alkaline phosphatase, and developed using an alkaline phosphatase color development buffer. Different positive control microsomes were supplemented with the different CYP IgG (e.g., microsomes from acetone-treated rats for CYP2E1, microsomes from phenobarbital-treated rats for CYP2A6, CYP2B1, and CYP2C6, microsomes from 3-methylcholanthrene-treated rats for CYP1A, microsomes from clofibrate-treated rats for CYP4A11) to ensure there was proper binding of the antibody (data not shown). Semi-quantitative comparisons of the relative densities of the different bands were made using densitometric analysis using a Scion Image Computer Program (Scion Corporation, Frederick, MD). All Western blots were performed four times per antibody probed using different sets of microsomes.

Analysis of Specific CYP Enzyme Activities in Microsomal Samples from Mice or Rats Pretreated with VCH, VCH-1,2-Epoxide, or VCD. Microsomal protein samples from mice or rats, pretreated as described as under *Materials and Methods*, were compared by their ability to biotransform model substrates of different CYP isoforms. Ethoxyresorufin has been used for measuring mouse CYP1A1 activity (Dickerson et al., 1999), rat CYP1A1 activity (Hashemi et al., 2000), and human hepatic CYP1A2 activity (Hengstler et al., 1997). Likewise, pentoxyresorufin has served as a model substrate for measuring CYP2B activity in different species, including mouse CYP2B9/10 (Posti et al., 1999), rat CYP2B1/2 (McKim et al., 1999), and human CYP2B6 (Gervot et al., 1999). The procedure of Lubet et al. (1985) was used for assessing the metabolism of ethoxy- or pentoxyresorufin.

Conversion of coumarin to 7-hydroxycoumarin was used to assess CYP2A activity. Methods have been previously described by Waxman and Walsch (1982). Honkakoski and Negishi (1997) have classified the CYP2A subfamily

TABLE 1

Comparison of epoxide formation from VCH in microsomes from female B6C3F$_1$ mice[a] or Fischer-344 rats treated with either 7.5 mmol/kg VCH i.p. for 10 days, or 80 mg/kg i.p. PB for 5 days

Metabolite	Treatment Group	Mouse	Rat
		nmol/mg microsomal protein/60 min	
VCH-1,2-epoxide	control	54.0 ± 8.0[b]	28.3 ± 2.8
	phenobarbital	318.6 ± 8.7 (5.9)[c]	79.3 ± 7.1 (2.8)
	VCH	205.2 ± 36.5 (3.8)	56.1 ± 5.3 (2.0)
VCH-7,8-epoxide	control	36.6 ± 5.2	22.0 ± 5.3
	phenobarbital	75.7 ± 1.3 (2.1)	35.0 ± 3.3 (1.6)
	VCH	75.0 ± 5.0 (2.0)	39.6 ± 5.3 (1.8)
VCD	control	N.D.	N.D.
	phenobarbital	7.6 ± 0.9	N.D.
	VCH	6.3 ± 0.9	N.D.

N.D., not-detectable.
[a] $n = 4$ groups of microsomes were pooled from mice (4 mice/group, 16 mice total/treatment). Microsomes were prepared from individual rats (four rats per treatment).
[b] Values represent mean (±S.D.) nanomoles of epoxide formed per milligrams of protein after 60 min.
[c] Values in parenthesis represent significant ($p < 0.05$) fold-increase in epoxide metabolite formation over control microsomal incubations.

that described the catalytic specificities of rat CYP2A3, mouse CYP2A5, and human CYP2A6. All of these enzymes catalyze coumarin 7-hydroxylation.

Microsomal hydroxylation of *p*-nitrophenol was used to assess CYP2E1 activity (Tassaneeyakul et al., 1993). Methods have been previously described by Forkert et al. (1996).

Metabolism of VCH, VCH-1,2-Epoxide, or VCH-7,8-Epoxide in Supersomes Containing Human CYP1A2, CYP2A6, CYP2B6, CYP2C9, CYP2E1, CYP3A4, CYP4A11, or Aromatase. To investigate whether expressed individual human CYP proteins were capable of mediating the epoxidation of VCH and its monoepoxide metabolites, the Supersome system (GENTEST, Woburn, MA) was used (human CYP + P450 reductase + cytochrome b_5). The 0.5-ml total incubation volume consisted of 50 pmol of CYP protein, 1 mM racemic VCH, and a recycling NADPH system (0.5 mM $NADP^+$, 1 unit/ml G6PDH, and 10 mM G6P). Samples were incubated at 37°C for up to 30 min. Epoxide metabolites were identified and quantified using gas chromatography. Blank incubations lacked G6P. Substrates were also incubated with control insect microsomes to ensure there was no metabolism without CYP enzymes.

Statistical Analysis. Student's *t* test was used to compare between means of two different samples. Data were considered significantly different at $p < 0.05$.

Results

Effects of VCH Pretreatment on In Vitro Metabolism of VCH, VCH-1,2-Epoxide, and VCH-7,8-Epoxide in Mouse and Rat Microsomes. Hepatic microsomes from untreated mice formed greater amounts of VCH-1,2-epoxide and VCH-7,8-epoxide compared with rats (Table 1). Incubations were conducted for up to 60 min to allow possible VCD formation, but no formation was detected in microsomes obtained from either the control mice or rats. Pretreatment of mice or rats with VCH caused 3.8- and 2.0-fold increases in the formation of VCH-1,2-epoxide in hepatic microsomes, respectively. VCH pretreatment also resulted in a 2.0-fold increase in VCH-7,8-epoxide formation by mouse hepatic microsomes and a 1.8-fold increase by rat hepatic microsomes. Only in the mouse did VCH pretreatment result in microsomal formation of VCD. Microsomes from phenobarbital-pretreated mice and rats exhibited 5.9- and 2.8-fold increases in VCH-1,2-epoxide formation, respectively, and 2.1- and 1.6-fold increases in VCH-7,8-epoxide formation, respectively. There were no differences in VCH metabolism between microsomes from nontreated animals and those that received vehicle; therefore, vehicle data is not shown.

Effects of VCH, VCH-1,2-Epoxide, or VCD Exposure on Total Cytochrome P450 Levels in Hepatic Microsomes from Mice or Rats. There were significant increases in total hepatic microsomal CYP levels in the mouse following repeated exposure to VCH or VCH-1,2-epoxide compared with nontreated mice (Table 2). Neither VCH, VCH-1,2-epoxide, or VCD caused significant increases in total CYP levels in the rat compared with control. As expected, PB caused significant increases in CYP levels in both species.

Effects of VCH Exposure on Specific Cytochrome P450 Levels in Microsomes from Mice or Rats. The levels of specific hepatic CYP enzymes in mice and rats exposed to VCH, VCH-1,2-epoxide, or VCD were examined with polyclonal antibodies to human CYP2E1, rat CYP1A1, rat CYP2A6, rat CYP2B1, rat CYP2C6, rat CYP3A2, and rat CYP4A1 (Fig. 2; Tables 3 and 4). All Western blots were performed four times per antibody probed using different sets of microsomes. The protein controls for the different CYP isoforms resulted in appropriate positive staining (data not shown). In the mouse, there were 2.5-fold and 1.9-fold increases in CYP2B protein levels following treatments with VCH or VCH-1,2-epoxide, respectively. There was also a 2.1-fold increase in CYP2A protein levels with VCH pretreatment in the mouse. There were not significant increases in CYP2A or CYP2B levels in the rat with either VCH, VCH-1,2-epoxide, or VCD pretreatment. Instead, CYP2A expression was decreased with VCH pretreatment, and CYP2B expression was decreased with VCH-1,2-epoxide or VCD pretreatment. There were no increases in CYP1A or CYP4A levels in microsomes from either species pretreated with VCH. Pretreatment with VCD caused elevated levels of CYP1A and CYP2C in the rat.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP1A Activity in Microsomes from Mice or Rats. To assess the effect of repeated treatment with VCH, VCH-1,2-epoxide, or VCD on the activity of CYP1A, the hepatic dealkylation of ethoxyresorufin was measured (Fig. 3). After repeated treatment with VCH or VCH-1,2-epoxide, small but significant increases of ethoxyresorufin dealkylation were observed in the mouse but not the rat. There were also significant increases in ethoxyresorufin dealkylation in the microsomes from mice pretreated with PB.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2A Activity in Hepatic Microsomes from Mice or Rats. Repeated exposure to VCH or VCH-1,2-epoxide significantly increased CYP2A activity in mouse hepatic microsomes, as measured by coumarin 7-hydroxylation (Fig. 4). Pretreatment of rats with VCH, VCH-1,2-epoxide, or VCD did not result in increased hepatic microsomal activity with respect to 7-hydroxylation of coumarin. Phenobarbital pretreatment increased coumarin hydroxylation in microsomes obtained from both species.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2B Activity in Hepatic Microsomes from Mice or Rats. The hepatic microsomal dealkylation of pentoxyresorufin, a

TABLE 2

Total cytochrome P450 levels in hepatic microsomes from VCH, VCH-1,2-epoxide-, VCD-, or phenobarbital-treated B6C3F$_1$ mice or Fischer-344 rats

	Total Cytochrome P450				
	Nontreated	VCH	VCH-1,2-epoxide	VCD	PB
	nmol/mg microsomal protein				
Mouse[a]	0.89 ± 0.08	1.31 ± 0.04[c]	1.48 ± 0.06[c]	1.05 ± 0.18	1.73 ± 0.10[c]
Rat[b]	0.91 ± 0.09	0.93 ± 0.12	1.00 ± 0.06	0.92 ± 0.08	1.64 ± 0.12[c]

[a] $n = 8$ groups of microsomes were pooled from mice (4 mice/group, 16 mice total/treatment). Microsomes were prepared from individual rats (four rats per treatment).
[b] $n = 8$ separate rats.
[c] Significantly different ($p < 0.05$) from control.

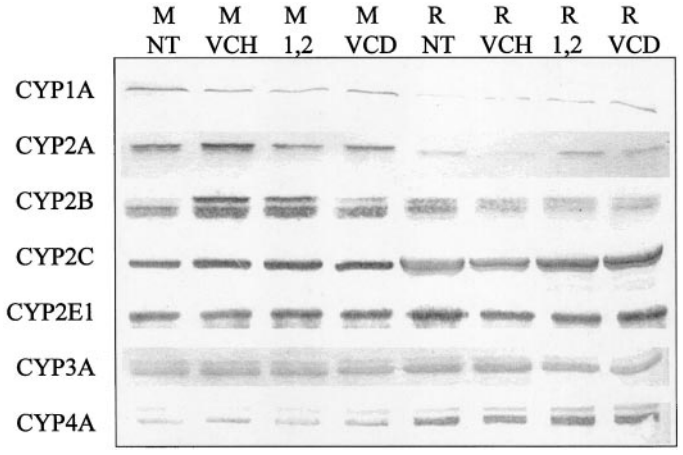

FIG. 2. *Western blots of hepatic microsomes isolated from VCH-1,2-epoxide- or VCD-treated B6C3F$_1$ mice and Fischer-344 rats.*

Mice and rats were treated as described under *Materials and Methods*. Microsomal proteins (1 or 10 μg) were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose filters, and probed with polyclonal antibodies raised to purified rat or human CYP enzymes. Immunoblots were quantitated by densitometric analysis (Tables 3 and 4). Microsomes were prepared from pooled livers from mice (4 mice/group, 16 mice/treatment) or individual rats (four rats per treatment). All Western blots were performed four times per antibody probed using different sets of microsomes. M, mouse; R, rat; NT, nontreated; 1,2, VCH-1,2-epoxide.

TABLE 3

Densitometry of Western blotting experiments

Densitometric analyses of Western blots (Fig. 2) using microsomes from B6C3F$_1$ mice treated with VCH (7.5 mmol/kg i.p. for 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days), as previously described under *Materials and Methods*. Densitometric analyses have been normalized to control (i.e., nontreated mice). Blots represent pooled microsomes from mice (four mice per group). All Western blots were performed four times per antibody probed using different sets of microsomes.

Treatment	Fold of Control						
	1A	2A	2B	2C	2E	3A	4A
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VCH	0.8	2.1	2.5	1.2	1.0	1.3	1.3
VCH-1,2-epoxide	0.7	1.0	1.9	1.4	1.1	1.3	1.1
VCD	1.0	1.2	1.3	1.0	1.0	1.2	1.3

model substrate of CYP2B, was enhanced by pretreatment of mice with either VCH or VCH-1,2-epoxide (Fig. 5). VCH pretreatment resulted in a 4.1-fold increase of pentoxyresorufin dealkylation over control, whereas VCH-1,2-epoxide caused a 2.2-fold increase over control. No increases in pentoxyresorufin dealkylation were observed in microsomes from rats pretreated with VCH and VCH-1,2-epoxide. VCD pretreatment caused a small but significant decrease in pentoxyresorufin dealkylation in rat hepatic microsomes. Phenobarbital pretreatment caused large increases in pentoxyresorufin dealkylation in both species.

TABLE 4

Densitometry of Western blotting experiments

Densitometric analyses of Western blots (Fig. 2) using microsomes from Fischer-344 rats treated with VCH (7.5 mmol/kg i.p. for 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days), as previously described under *Materials and Methods*. Densitometric analyses have been normalized to control (i.e., nontreated rats). Blots represent microsomes from individual rats (four rats per treatment group). All Western blots were performed four times per antibody probed using different sets of microsomes.

Treatment	Fold of Control						
	1A	2A	2B	2C	2E	3A	4A
None	1.0	1.0	1.0	1.0	1.0	1.0	1.0
VCH	1.1	0.5	0.7	0.9	0.8	1.0	0.9
VCH-1,2-epoxide	1.9	1.5	0.6	1.3	0.9	1.1	1.2
VCD	2.3	1.1	0.7	1.7	1.0	1.0	1.2

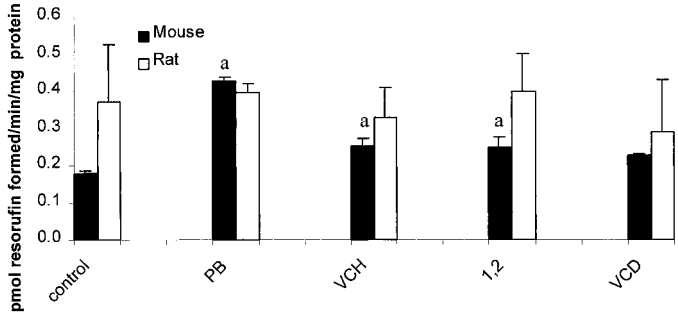

FIG. 3. *Ethoxyresorufin dealkylation activity in hepatic microsomes from VCH-, VCH-1,2-epoxide-, VCD-, or PB-treated B6C3F$_1$ mice and Fischer-344 rats.*

Hepatic microsomes were isolated from mice or rats and administered PB (80 mg/kg i.p. for 5 days), VCH (7.5 mmol/kg i.p. for 5 or 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days). Microsomes (0.25 mg/ml) were incubated for 10 min with 10 μM ethoxyresorufin. Data represent the mean ± S.D. of either four individual rats or four groups of pooled microsomes from mice (four mice per treatment group). Statistically significant ($p < 0.05$) compared with control of same species (a). 1,2, VCH-1,2-epoxide.

Effects of Repeated Exposure to VCH, VCH-1,2-Epoxide, or VCD on CYP2E1 Activity in Hepatic Microsomes from Mice or Rats. The microsomal hydroxylation of *p*-nitrophenol, a model substrate of CYP2E1 (Tassaneeyakul et al., 1993), was not affected by pretreatment with either VCH, 1,2-epoxide, or VCD in either the mouse or the rat (Fig. 6). Previous studies with acetone-pretreated microsomes from mice or rats pretreated with the CYP2E1 inducer acetone (1% in drinking water for 5 days) demonstrated significant increases in *p*-nitrophenol hydroxylation in the mouse and rat compared with control (Fontaine et al., 2001).

Incubations with Supersomes Containing Specific Human CYP Enzymes. Purified protein systems were used to determine which human CYP isoforms are capable of catalyzing the epoxidation of VCH and its epoxide metabolites (Fig. 7, A and B). Of the isoforms tested, only human CYP2B6 and CYP2E1 showed substantial catalytic toward VCH in forming VCH-1,2-epoxide and VCH-7,8-epox-

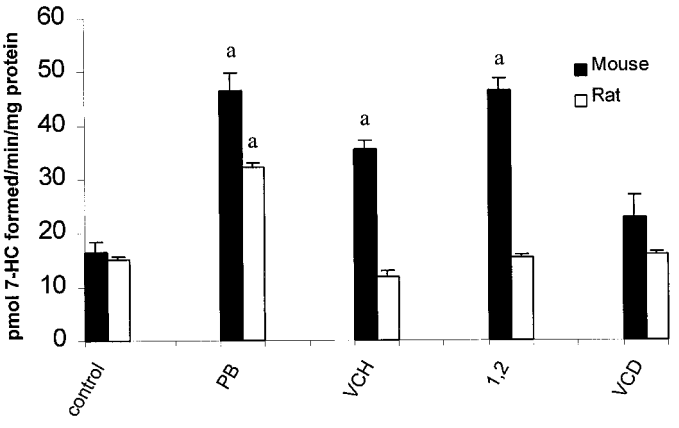

FIG. 4. *Coumarin hydroxylation activity in hepatic microsomes from VCH-, VCH-1,2-epoxide-, VCD-, or PB-treated B6C3F$_1$ mice and Fischer-344 rats.*

Hepatic microsomes were isolated from mice or rats and administered PB (80 mg/kg i.p. for 5 days), VCH (7.5 mmol/kg i.p. for 5 or 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days). Microsomes (0.1 mg/ml) were incubated for 15 min with 50 μM coumarin. Data represent the mean ± S.D. of either four individual rats or four groups of pooled microsomes from mice (four mice per treatment group). Statistically significant ($p < 0.05$) compared with control of same species (a). 1,2, VCH-1,2-epoxide.

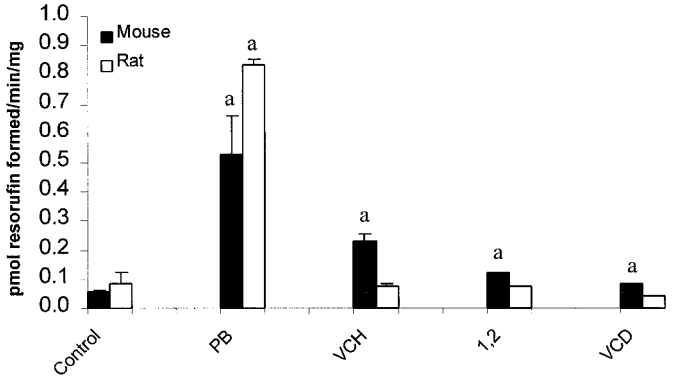

FIG. 5. *Pentoxyresorufin dealkylation activity in hepatic microsomes from VCH-, VCH-1,2-epoxide-, VCD-, or PB-treated B6C3F$_1$ mice and Fischer-344 rats.*

Hepatic microsomes were isolated from mice or rats and administered PB (80 mg/kg i.p. for 5 days), VCH (7.5 mmol/kg i.p. for 5 or 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days). Microsomes (0.25 mg/ml) were incubated for 10 min with 10 μM pentoxyresorufin. Data represent the mean ± S.D. of either four individual rats or four groups of pooled microsomes from mice (four mice per treatment group). Statistically significant ($p < 0.05$) compared with control of same species (a). 1,2, VCH-1,2-epoxide.

ide. The major product formed by CYP2B6 was VCH-7,8-epoxide, whereas the major product formed by CYP2E1 was VCH-1,2-epoxide. CYP2A6, CYP2B6, CYP2E1, and CYP3A4 were capable of catalyzing the epoxidation of both monoepoxides to form the diepoxide (Fig. 8, A and B).

Discussion

Although VCH is ovotoxic in female mice, female rats are resistant due to limitations in forming the epoxide metabolites (Smith et al., 1990a). Furthermore, repeated exposure to VCH is required to elicit this ovotoxicity in mice. This repeated exposure is associated with an increase in VCH bioactivation both in vivo and in vitro (Doerr-Stevens et al., 1999). Therefore, elevated protein levels and activities of the cytochrome P450 isoforms responsible for the bioactivation of VCH would be expected following repeated exposure of mice to VCH. Through chemical induction studies and examination of total

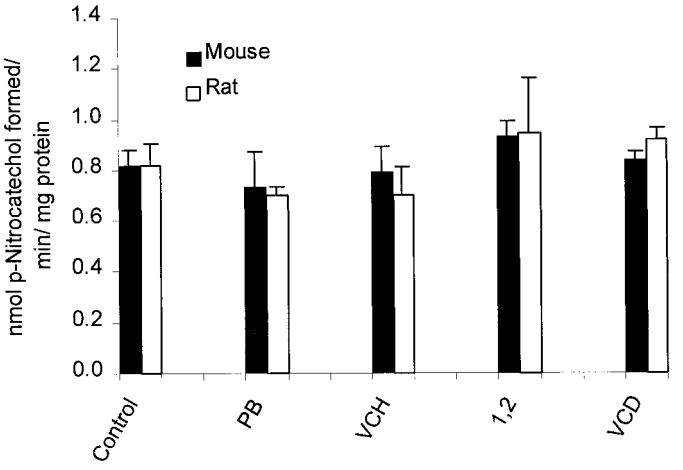

FIG. 6. *p-Nitrophenol hydroxylase activity in hepatic microsomes from VCH-, VCH-1,2-epoxide-, VCD-, or PB-treated B6C3F$_1$ mice and Fischer-344 rats.*

Hepatic microsomes were isolated from mice or rats and administered PB (80 mg/kg i.p. for 5 days), VCH (7.5 mmol/kg i.p. for 5 or 10 days), VCH-1,2-epoxide (1.75 mmol/kg i.p. for 10 days), or VCD (0.4 mmol/kg i.p. for 10 days). Microsomes were incubated for 10 min with 200 μM *p*-nitrophenol. Data represent the mean ± S.D. of either four individual rats or four groups of pooled microsomes from mice (four mice per group). Statistically significant ($p < 0.05$) compared with control of same species (a). 1,2, VCH-1,2-epoxide.

and specific CYP levels and activities following repeated exposure to VCH, the roles of at least two hepatic CYP isoforms in the metabolism of VCH have now been revealed.

There is substantial evidence for the role of bioactivation in the species-dependent ovotoxicity of VCH. Repeated exposure to VCH caused greater increases in VCH epoxidation to VCH-1,2-epoxide and VCD in the mouse compared with the rat. There were also significant increases in total hepatic CYP levels in these mice and in mice pretreated with VCH-1,2-epoxide, whereas neither of these pretreatments caused significant increases in total CYP levels in the rat. Although VCH-1,2-epoxide pretreatment resulted in slightly greater increases in total hepatic CYP levels than VCH, VCH-1,2-epoxide did not increase specific CYP levels tested to the extent as VCH. Therefore, other CYP isoforms may play a role in the biotransformation of VCH and/or VCH-1,2-epoxide. Since repeated treatment with VCH is required for ovotoxicity and the mouse forms more VCH-1,2-epoxide than the rat, this metabolite may contribute to the enhanced VCH bioactivation in the mouse.

The current study defined the roles of CYP2B and CYP2A in the species-dependent bioactivation of VCH. Significant increases in CYP2B levels were seen in microsomes from mice pretreated with VCH or VCH-1,2-epoxide. These data correlate with the increases in pentoxyresorufin dealkylation in microsomes from VCH and VCH-1,2-epoxide pretreated mice. In contrast, there were no increases in CYP2B levels or activity seen with pretreatments other than PB in the rat. Instead, these pretreatments appeared to decrease levels in rat hepatic microsomes. The associated CYP2B activity was also reduced in rats pretreated with VCD, further indicating the role of CYP2B in VCH metabolism.

There were also significant increases in CYP2A protein levels seen in microsomes from mice pretreated with VCH, and significant increases in coumarin 7-hydroxylation in microsomes from mice pretreated with either VCH or VCH-1,2-epoxide. In contrast, there were no increases in CYP2A levels or activity in microsomes by VCH, VCH-1,2-epoxide, or VCD pretreatment in rats. Again, these results correlate with other data that suggest why VCH pretreatment results in greater in vitro bioactivation of VCH in the mouse than in the rat.

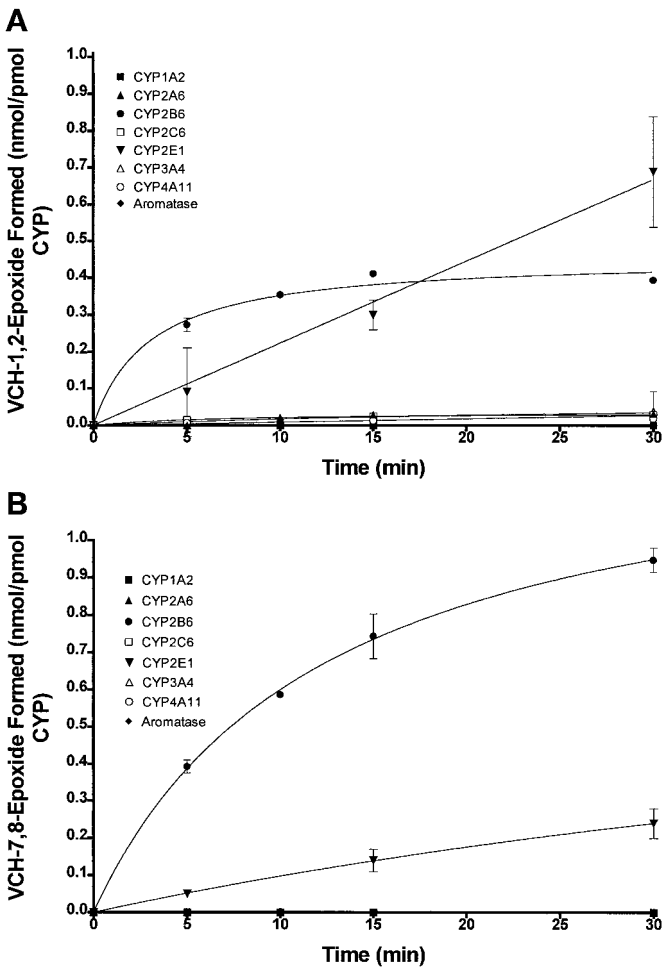

FIG. 7. *Comparison of VCH-1,2-epoxide and VCH-7,8-epoxide formation from VCH in Supersomes containing 100 nM (50 pmol) either human CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11, or aromatase.*

There was no detectable metabolism of VCH in forming VCH-7,8-epoxide by aromatase, CYP1A2, CYP2A6, CYP2C6, CYP3A4, or CYP4A11 after 30 min.

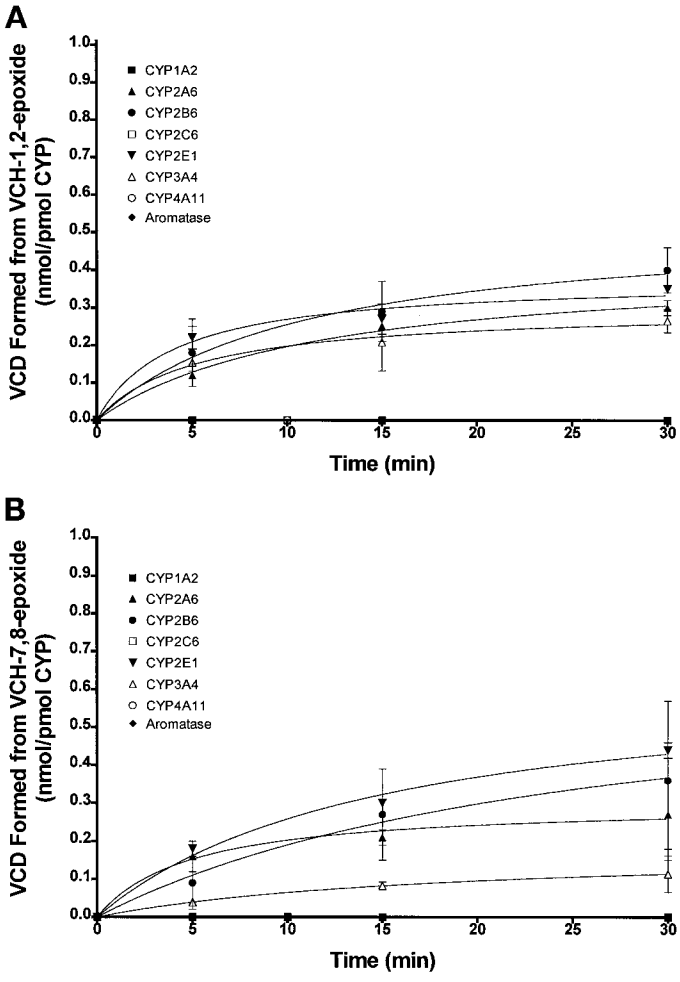

FIG. 8. *Comparison of VCD formation from VCH-1,2-epoxide or VCH-7,8-epoxide in Supersomes containing 100 nM (50 pmol) either human CYP1A2, CYP2A6, CYP2B6, CYP2C6, CYP2E1, CYP3A4, CYP4A11, or aromatase cDNA.*

There was no detectable metabolism of VCH-1,2-epoxide or VCH-7,8-epoxide by aromatase, CYP1A2, CYP2C6, or CYP4A11 after 30 min.

It is well known that PB is a potent inducer of CYP2A, CYP2B, and CYP3A in humans and rodents (Cheng and Schenkman, 1982; Gervot et al., 1999). Current studies show that pretreatment with PB resulted in significant increases in total hepatic CYP, CYP2A, and CYP2B activities and levels (data not shown) and up to 6-fold increases in VCH epoxidation in microsomes from mice and rats. Although PB is known to induce CYP3A, previous studies eliminated this subfamily as a critical hepatic CYP isoform in VCH bioactivation in the mouse. For example, anti-rat P450IIIA IgG inhibited testosterone 6β-hydroxylation by 68% but did not effect VCH epoxidase activity in murine microsomes (Smith et al., 1990c). Furthermore, CYP3A levels and activities were not significantly increased in the mouse following either 5- or 10-day pretreatment (Doerr-Stevens et al., 1999). Moreover, immunoblotting data and CYP3A4 Supersome metabolism described in current studies reveal that CYP3A does not contribute to VCH bioactivation.

Female human microsomes demonstrated epoxidation of VCH at rates 13-fold and 2-fold less than those in mice and rats, respectively (Smith and Sipes, 1991), and total hepatic CYP per milligram of protein is significantly lower in humans than in rodents (Imaoka et al., 1991; Shimada et al., 1994). Interestingly, the eight human hepatic CYP isoforms tested in current studies, CYP2E1 and CYP2B6, were the only isoforms that significantly catalyzed the epoxidation of VCH. Previous experiments focused on the role of CYP2E1 in the epoxidation of VCH because it has been reported to metabolize the structurally related compounds styrene and 1,3-butadiene (Lieber, 1997; Nieusma et al., 1998; Fontaine et al., 2001). Studies showed that, although hepatic microsomes from mice and rats pretreated with acetone showed increases in VCH-1,2-epoxide formation from VCH, hepatic microsomes from mice or rats pretreated with VCH for 5 or 10 days demonstrated no increases in CYP2E1 protein levels or activity (Fontaine et al., 2001). Those data, combined with the data showing no differences in epoxidation of VCH or its monoepoxides in CYP2E1-deficient mouse hepatic microsomes compared with those of mice that do have CYP2E1, indicated that CYP2E1 is not an important isoform in the species-specific bioactivation of VCH. Current studies reconfirm this conclusion because neither VCH, VCH-1,2-epoxide, nor VCD pretreatment for 10 days affected CYP2E1 levels or activity in mice or rats.

Interestingly, although CYP2B6 and CYP2E1 were the only CYP isoforms that catalyzed VCH epoxidation in humans, CYP2B6, CYP2A6, CYP2E1, and CYP3A4 catalyzed the epoxidation of both monoepoxides to form the diepoxide. Although CYP3A4 is the major hepatic CYP isoform in humans, human liver CYP2A6 expression is relatively low (approximately 4%) (Cheng and Schenkman, 1982; Shimada et al., 1994). However, since the rate of formation of VCH-

1,2-epoxide was shown to be significantly limited in humans compared with the mouse or rat (Smith and Sipes, 1991), the possibility of VCH-1,2-epoxide or VCH-7,8-epoxide bioactivation to VCD by these particular enzymes is unlikely in humans exposed to VCH.

In conclusion, comparisons of VCH metabolism and hepatic CYP induction demonstrate that CYP2A and CYP2B are important CYP isoforms in the species-dependent bioactivation and, therefore, ovotoxicity of VCH. The increased expression of CYP2A and CYP2B seen exclusively in the mouse appears to be due to repeated treatment with VCH or VCH-1,2-epoxide. This indicates that, with repeated exposure to VCH, the mouse is exposed to a greater concentration of the ovotoxic metabolites via enhanced bioactivation. The rat is resistant to the ovotoxicity of VCH, at least in part, because the increases in CYP levels/activities do not occur following repeated exposure to VCH. It is not known if exposure of humans to VCH would result in elevated levels of CYP isoforms. Perhaps studies with cultures of human hepatocytes could help address this question.

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