INDUCTION OF CYTOCHROME P-450 ENZYMES AFTER REPEATED EXPOSURE TO
4-VINLYCycLOHEXENE IN B6C3F1, MICE

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ABSTRACT:

4-Vinylcyclohexene (VCH), an ovarian toxicant in mice, is known to irreversibly deplete ovarian follicles as a consequence of VCH diepoxide formation. Because ovotoxicity requires repeated dosing of VCH, the effect of consecutive daily doses of VCH (7.5 mmol/kg/day) on mouse liver microsomal activities and VCH epoxidation was determined. Cytochromes P-450 2B and 2A (CYP2B and CYP2A), principle isoforms involved in the bioactivation of VCH, as well as CYP2E1 and CYP3A were evaluated. VCH exposure increased total cytochrome P-450 content (35–83% above control levels) after either 5, 10, or 15 days of treatment. Western blot analysis revealed an induction of CYP2A, CYP2B, and CYP2E1 at day 10. Elevated levels of CYP2A and CYP2B correlated with marker androstenedione and testosterone 16α- and 16β-hydroxylase activities. Microsomes prepared from mice pretreated with VCH for 10 days demonstrated an increase (≥2-fold) in the rate of VCH monoepoxide and diepoxide formation. Microsomal VCH epoxidation was increased to a similar extent by phenobarbital, acetone, and dexamethasone treatment. An increase in cytosolic glutathione-S-transferase activity was observed after repeated VCH treatment, an enzyme potentially involved in detoxification of the VCH epoxides. Interestingly, preliminary studies indicated that circulating levels of the monoepoxide (vinylcyclohexene 1,2-monoepoxide) and diepoxide of VCH were elevated after repeated dosing of VCH. Overall, the results indicate that repeated exposure of VCH in mice induces cytochrome P-450-dependent activities, and in turn induction of its metabolism. Additional studies examining the toxicokinetics of VCH after repeated exposure are required to further delineate the relevance of induction in VCH-induced ovotoxicity.

4-Vinylcyclohexene (VCH) is an industrial compound used as an intermediate in chemical production and produced as a by-product in butadiene processing (International Agency for Research on Cancer, 1994). Exposure to this compound is of toxicological significance, as animal studies have revealed that VCH is an ovarian toxicant and carcinogen in B6C3F1 mice. VCH depletes preantral ovarian follicles after repeated exposure by a number of routes (Collins and Manus, 1987; Smith et al., 1990b; Bevan et al., 1996). VCH-induced follicular loss is irreversible, resulting in premature ovarian failure (Hooser et al., 1994). In addition, follicular loss is temporally related to the formation of preneoplastic lesions (Hooser et al., 1994). These early ovarian changes may be associated with the increased incidence of VCH-induced ovarian neoplasms, including mixed benign tumors, granulosa cell tumors, and granulosa cell carcinomas (National Toxicology Program, 1986; Collins et al., 1987).

Interestingly, ovarian neoplasms as well as follicular loss occur in B6C3F1 mice but not in Fischer 344 rats after exposure to VCH (National Toxicity Program, 1986; Smith et al., 1990b). This species variation most likely relates to differences in the biotransformation of VCH to ovotoxic epoxides. Female mice metabolize VCH to epoxides to a greater extent than female rats (Smith et al., 1990a). Although VCH is not ovotoxic in rats, administration of the epoxides of VCH to rats results in a significant depletion of ovarian follicles (Smith et al., 1990b). In addition, inhibition of VCH metabolism in mice results in partial protection from VCH-induced ovarian injury (Smith et al., 1990b). Although VCH may be metabolized to a number of ovotoxic epoxides, the diepoxide of VCH (VCD) is the most potent ovarian toxicant (Smith et al., 1990b), and structure-activity studies indicate that VCD is the ultimate ovotoxic metabolite of VCH (Doerr et al., 1995).

Data indicate that the liver is the major site of bioactivation of VCH. VCH is metabolized to either vinylcyclohexene 1,2-monoepoxide (1,2-VCHE) or vinylcyclohexene 7,8-monoepoxide (7,8-VCHE) in murine hepatic microsomes (Fig. 1; Smith et al., 1990a).
These monoepoxide metabolites are further oxidized in vitro to form VCD (Keller et al., 1997). In vitro metabolism of VCH to VCD in hepatic microsomes prepared from phenobarbital-treated mice has been demonstrated (Gervasi et al., 1980). In comparison, undetectable levels of the epoxides of VCH were noted after incubation of VCH and the monoepoxides in ovarian microsomes isolated from naïve mice (Keller et al., 1997). In addition, circulating levels of 1,2-VCHE and 7,8-VCHE, and the metabolism of 1,2-VCHE to VCD were determined by the method of Omura and Sato (1964) with the extinction coefficient of 21 mM$^{-1}$cm$^{-1}$, which corresponds to 1 mM$^{-1}$cm$^{-1}$.

The major cytochrome P-450 (P-450) isoforms involved in the epoxidation of VCH to 1,2-VCHE in untreated female B6C3F1 mice are cytochromes P-450 2A and 2B (CYP2A and CYP2B) (Smith et al., 1990c). CYP3A was shown not to be involved in the epoxidation of VCH to 1,2-VCHE (Smith et al., 1990b). Thus, mice were treated with a single dose of VCH (7.5 mmol/kg/day i.p.) for a varying number of days; and the effect on total P-450 content, specific P-450-dependent activities, immunoreactive P-450 isozyme levels, glutathione (GSH) S-transferase (GST) activity, as well as the in vitro oxidation of VCH was determined. Studies were designed to evaluate CYP2A, CYP2B, CYP3A, and CYP2E1.

Materials and Methods

**Chemicals.** VCH and 1,2-VCHE were purchased from Aldrich Chemical Co. (Milwaukee, WI). VCD and phenobarbital (PB) were obtained from Pfaltz and Bauer, Inc. (Waterbury, CT) and Mallinckrodt Specialty Chemicals (Chesterfield, MO), respectively. 7,8-VCHE was synthesized by the Synthetic Chemistry Core of the Environmental Health Sciences Center. VCH and the VCD epoxides were racemic with chemical purities of 98% or greater. Dexamethasone (DEX), cytochrome c (equine heart), NADPH (tetrasodium salt), β-NADP$^+$ (sodium salt), glucose 6-phosphate (monosodium salt), glucose 6-phosphate dehydrogenase (type XV), and GSH were purchased from Sigma Chemical Co. (St. Louis, MO). $^{14}$C-Testosterone (57 mCi/mmol) and $^{14}$C-androstenedione (53.86 mCi/mmol) were obtained from Amersham (Arlington Heights, IL) and DuPont-New England Nuclear (Boston, MA), respectively. Other chemicals were of reagent grade. (Caution: VCH and its epoxides are either potential carcinogens or are carcinogens in animals and should be handled with appropriate precautions.)

**Animals.** Female B6C3F1 mice were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN). The mice were housed five per cage in a biohazard hood, provided food (4% mouse/rat diet; Harlan Teklad, Madison, WI) and water ad libitum, and maintained on a 12-h light/dark cycle in a controlled temperature of 22 ± 2°C. The animals were acclimated to this environment for 7 days before use in studies. All animals were euthanized by inhalation of CO$_2$ after various treatments.

**Subcellular Preparations and Characterization.** Mouse livers were removed and microsomes and cytosol were isolated by differential ultracentrifugation as described by Guengerich (1989). Protein concentration was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL) with bovine serum albumin as a standard. Microsomal P-450 content was determined by the method of Omura and Sato (1964) with the extinction coefficient 91 mM$^{-1}$cm$^{-1}$. P-450 reductase activity was estimated by the rate of cytochrome c reduction (Phillips and Langdon, 1962). Samples consisted of (final concentrations) murine hepatic microsomes (25 μg/ml), 50 mM cytochrome c, 0.12 mM NADPH, and 0.3 M potassium phosphate buffer (pH 7.7) made up to a final volume of 1 ml. The blank consisted of all of the components, except NADPH. Absorbance at 550 nm was measured every 10 s over a 5-min period. The extinction coefficient of 21 mM$^{-1}$cm$^{-1}$, which corresponds to 1 μmol of cytochrome c/ml at 550 nm, was used to determine nanomoles of cytochrome c reduced per minute per milligram of protein.

**VCH and 1,2-VCHE In Vitro Metabolism.** The metabolism of VCH to 1,2-VCHE and 7,8-VCHE, and the metabolism of 1,2-VCHE to VCD were assessed in hepatic microsomes isolated from mice after treatment with VCH or various P-450 inducers.

**Animal treatments.** Female mice (44–47 days old, 16–23 g) received either VCH (7.5 mmol/kg/day x 10 days i.p.); sesame seed oil (vehicle control, 2.5 ml/kg/day x 10 days i.p.); PB (80 mg/kg/day x 5 days i.p.); DEX (100 mg/kg/day x 3 days i.p.), or acetone (ACE) [in drinking water 1% (v/v) x 5 days]. PB was made up as a 3.2% solution (w/v) in 0.9% NaCl, and DEX was in a 2% TWEEN 80 solution (w/v). Animals were allowed free access to food and water throughout the injection periods. Hepatic microsomes were isolated 24 h after the last treatment for each group. The microsomes were prepared with four livers in each pool, a total of three to eight individual pools for each treatment.
Incubation procedures. Microsomal incubations contained (final concentrations) 1 mM VCH or 1,2-VCHE in either methanol or acetone (1% v/v), 0.75 mg of microsomal protein/ml, 0.5 mM NADP⁺, 10 mM glucose 6-phosphate, 1 U of glucose 6-phosphate dehydrogenase, 50 mM HEPEs (pH 7.6), 0.1 mM EDTA, and 15 mM MgCl₂ at a final volume of 1 ml. Samples were precoated in a shaking water bath at 37°C for 3 min. Reactions were initiated with the addition of glucose 6-phosphate and incubated at 37°C for an additional 10 min. Glucose 6-phosphate was absent from blank reactions. Reactions were terminated by immersing the incubation vials in liquid nitrogen before placing in ice. The epoxide metabolites of VCH were extracted from the samples into 240 μl of ethyl acetate by vortexing and then shaking for 10 min. The phases were separated by centrifugation (10 min, 3000 rpm). The organic layer was removed after freezing of the sample at −80°C and analyzed for 1,2-VCHE and 7,8-VCHE or VCD by gas chromatography and gas chromatography/mass spectrometry. The extraction efficiencies of 1,2-VCHE, 7,8-VCHE, and VCD were 96%, 93%, and 70%; respectively. Reported values were corrected for recovery.

**VCH In Vivo Metabolism.** Metabolism of VCH to 1,2-VCHE and VCD was determined in vivo in 43- to 60-day-old female mice. Animals received either a single or multiple doses of VCH as indicated below. Dose selection of VCH was based on previous ovotoxicity studies conducted in our laboratory (Smith et al., 1990b).

*Animal treatments.* VCH (7.5 mmol/kg i.p.) or sesame seed oil (2.5 ml/kg i.p.) was administered to mice for either 5, 10, or 15 days (N = 5/group). At day 6, 11, or 16 a challenge dose of VCH (7.5 mmol/kg i.p.) was given to both groups, and epoxide blood levels were determined 1 h after administration of the challenge dose. [Note: A 1-h time interval for blood collection was chosen because it was at this point after administration of VCH for 10 days (7.5 mmol/kg i.p.) that maximum circulating levels of 1,2-VCHE and VCD were noted (data not shown)]. To demonstrate that repeated doses of VCH did not lead to in vivo accumulation of the VCH epoxides, additional groups of animals received VCH (7.5 mmol/kg i.p.; N = 5/group) for either 5, 10, or 15 days, but did not receive the challenge dose on day 6, 11, or 16; instead epoxide blood levels were determined 24 h after the last dose. Animals were sacrificed by CO₂ inhalation at the designated time points, and blood was drawn from the posterior vena cava into heparinized syringes. The blood samples were extracted as described (refer to VCH and 1,2-VCHE In Vitro Metabolism) and analyzed by gas chromatography and gas chromatography/mass spectroscopy for 1,2-VCHE and VCD. Livers were also obtained at this time from each group, frozen in liquid nitrogen, and stored at −80°C.

*Enzyme assays.* Hepatic cytosol and microsomes were prepared from individual animals for each treatment group of the VCH in vivo metabolism study (N = 5 mice/treatment group; preparations were not pooled). Enzyme assays were conducted utilizing these fractions as indicated below.

*Steroid hydroxylase.* The oxidative metabolism of testosterone and androstenedione was determined by the method of Waxman (1991). Incubations contained (final concentrations) 25 μM 14C-testosterone or 14C-androstenedione (0.02 μCi/μmol), 25 μg of microsomal protein, 50 mM HEPEs buffer (pH 7.6), 15 mM MgCl₂, 0.1 mM EDTA, and 1 mM NADPH in a final volume of 100 μl. After a 2-min preincubination at 37°C, the reactions were initiated by the addition of NADPH and terminated after 15 min with either ethyl acetate (1 ml, testosterone assays) or trihydrofluoran (50 μl, androstenedione assays). The samples were vortexed and centrifuged (3000 rpm, 5 min). For androstenedione samples, 50-μl aliquots were applied to a thin-layer chromatography plate [Si250F (19c); J. T. Baker, Phillipsburg, NJ] directly and developed twice in 1 mM 14 C-testosterone or 14 C-androstenedione was determined by the method of Waxman (1991). Incubations contained (final concentrations) 1 mM 1-chloro-2,4-dinitrobenzene in ethanol, 1 mM GSH, 0.01 mg of cytosolic protein/ml, and 0.1 M potassium phosphate buffer (pH 6.5) to a final volume of 1 ml. Reactions were initiated by the addition of GSH and incubated at 37°C for 5 min. Reactions were terminated by the addition of methanol (1 ml) and the absorbance at 340 nm was determined. The molar extinction coefficient was 9.6 mM⁻¹ cm⁻¹.

*Analytical Methods.* The gas chromatography and gas chromatography/mass spectroscopy analytical methods developed for quantification of 1,2-VCHE, 7,8-VCHE, and VCD were as described in Doerr et al. (1995) with the following modifications. Injection volume of ethyl acetate extracts was 2 μl. The initial oven temperature was held at 75°C for 10 min, and then ramped to 230°C at a rate of 15°C/min and held at the final temperature for 4 min. The retention times for 1,2-VCHE, 7,8-VCHE, and VCD were 18 min, 19 min, and 22 min, respectively.

**Immunobots.** Western blot analysis of the murine hepatic microsomal proteins utilized for the VCH and 1,2-VCHE in vitro metabolism studies were prepared. Separation of microsomal proteins was performed by SDS-polyacrylamide gel electrophoresis (PAGE) with a 7.5% acrylamide gel (Laemmli, 1970). Proteins were electrophoretically transferred to nitrocellulose (Towbin et al., 1979), and then were blocked with 3% bovine serum albumin (fraction V) in TTBS (500 mM NaCl/20 mM Tris base/0.1% Tween 20) for 30 min, followed by incubation with the primary antibody for 1 h. The filter was then incubated with alkaline phosphate-conjugated goat anti-rabbit IgG for 1 h. Blots were developed with 5-bromo-4-chloro-3-indolyl-phosphate, p-toluidine salt, and nitro blue tetrazolium. Band intensities were quantified with an AMBIS 4000 image detector (AMBIS, Inc., San Diego, CA). Primary antibodies to rat CYP2B1 (Duignan et al., 1987) and CYP3A2 (Graves et al., 1987) were used. Anti-rat-CYP2E1 IgG was purchased from Amersham. Anti-rat-CYP2A IgG was a generous gift from Dr. Michael Murray of Westmead Hospital (Westmead, Australia).

**Statistical Analysis.** Student’s t test was used to compare means of two different samples. Comparisons between multiple groups were made with a one-way analysis of variance. When appropriate, significance was determined using the Student-Newman-Keuls t test. Data were considered significantly different at p < .05.

**Results**

Effect of VCH Treatment on VCH and 1,2-VCHE Hepatic Microsomal Metabolism. The rates of formation of the epoxides of VCH were elevated in microsomes prepared from animals administered repeated doses of VCH. A 2.6-fold and 2.2-fold increase in the rate of conversion of VCH to 1,2-VCHE and 7,8-VCHE was observed in hepatic microsomes from 10-day VCH-treated mice (7.5 mmol/kg/day i.p.; Table 1), respectively. Similarly, the metabolism of 1,2-VCHE to VCD was 2.1-fold higher in the VCH-treated microsomes, as compared to metabolism observed in nontreated microsomes (Table 2). Microsomal P450 levels were increased by 45 to 65% in VCH-treated mice, as compared to nontreated mice (Tables 1 and 2).

The level of enhancement of 1,2-VCHE epoxidation observed for VCH was similar to that observed with classical inducers of P-450. Treatment of mice with either ACE, PB, or DEX resulted in a 2.0- to 3.6-fold increase in the microsomal metabolism of 1,2-VCHE to VCD and a 36 to 82% increase in P450 (Table 2).

**Effect of VCH Treatment on Hepatic Microsomal Steroid Hydroxylase Activities.** To assess the effect of repeated exposure to VCH on the activity of specific P450 isoforms, the hepatic metabolism of androstenedione and testosterone was determined (Figs. 2 and 3). To investigate the activity of CYP2B, hydroxylation of androstenedione and testosterone in the 16α and 16β positions was determined (Harada and Negishi, 1984a; Honkakoski et al., 1992). Testosterone hydroxylation in the 15α and 16β positions was used to assess CYP2A and CYP3A activity, respectively (Harada and Negishi, 1984b; Wrighton et al., 1985).

After 5 days of repeated treatment with VCH (7.5 mmol/kg/day i.p.), significant increases were observed in the microsomal hydroxylation of androstenedione at the 16α (1.3- to 1.7-fold) and 16β (1.9- to 2.7-fold) positions (Fig. 2). Further increases in the formation of these metabolites were noted after 10 and 15 days of treatment with
TABLE 1
Epoxidation of VCH in hepatic microsomes isolated from B6C3F1, mice treated with multiple doses of VCH

Animals were treated with 7.5 mmol of VCH/kg/day i.p. for 10 days, and livers were harvested for microsomal preparation 24 h following the last dose. Microsomes (0.75 mg of protein) were incubated for 10 min with 1 mM VCH.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Epoxide Metabolite</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1,2-VCHE</td>
<td>7,8-VCHE</td>
</tr>
<tr>
<td>None</td>
<td>5 ± 1.8</td>
<td>2.6 ± 0.5</td>
</tr>
<tr>
<td>VCH</td>
<td>11.0 ± 1.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>5.9 ± 1.1&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>DEX</td>
<td>2.0 ± 0.2</td>
<td>2.0 ± 0.2</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent the mean ± S.D. of triplicate incubations using three different sets of pooled microsomes.

<sup>b</sup> Statistically different from control group (p < .0001).

TABLE 2
Epoxidation of 1,2-VCHE in hepatic microsomes isolated from B6C3F1, mice treated with different inducing agents

Animals were treated with 7.5 mmol of VCH/kg/day i.p. for 10 days or with inducing agents as described in Materials and Methods, and livers were harvested for microsomal preparation 24 h following the last dose. Microsomes (0.75 mg of protein) were incubated for 10 min with 1 mM 1,2-VCHE.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>VCD&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Fold Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>1.1 ± 0.1</td>
<td>2.1</td>
</tr>
<tr>
<td>VCH</td>
<td>1.6 ± 0.1</td>
<td>2.1</td>
</tr>
<tr>
<td>ACE</td>
<td>1.5 ± 0.1</td>
<td>2.0</td>
</tr>
<tr>
<td>PB</td>
<td>1.7 ± 0.1</td>
<td>3.6</td>
</tr>
<tr>
<td>DEX</td>
<td>2.0 ± 0.1</td>
<td>2.8</td>
</tr>
</tbody>
</table>

<sup>c</sup> Data represent the mean of two experiments, each performed in triplicate with pooled microsomes. Relative S.D. was <8%.

VCH, and these rates were significantly greater than those observed after 5 days of treatment with VCH (p < .05; Fig. 2).

An increase in the metabolism of testosterone in the 16α and 16β positions was observed after 5, 10, and 15 days of treatment with VCH (Fig. 3). Repeated dosing with VCH also increased hydroxylation of testosterone in the 15α position by 2-fold. No differences in 6β-hydroxylase activity were noted in VCH-treated groups, except for a slight but significant increase in microsomes prepared from animals exposed to VCH for 15 days (Fig. 3).

Interestingly, administration of a challenge dose of VCH 1 h before microsomal isolation resulted in complete attenuation of testosterone 15α-hydroxylase activity (Fig. 3). In the same treatment group, a partial attenuation of the enhanced 16α and 16β microsomal hydroxylase activities was also observed; however, this effect was not consistently noted (Figs. 2 and 3). These results parallel the effects observed on P-450 content. The VCH group (VCH+) that received a challenge dose 1 h before blood and liver collection had a slightly, but significantly, lower hepatic P-450 level compared to the VCH group (VCH−) that did not receive this challenge dose (Table 3).

**Effect of VCH Exposure on Expression of Select Hepatic P-450 Isoforms.** The levels of specific isozymes of hepatic P-450 were examined with polyclonal antibodies to rat CYP2A, CYP2B1, CYP2E1, and CYP3A2. P-450 isoform expression was also determined in microsomes isolated from ACE−, PB−, and DEX-treated mice. Induction of hepatic P-450 isoforms by these inducers was consistent with that previously reported (Fig. 4; Table 4; Freeman et al., 1992; Honkakoski and Lang, 1989; Meehan et al., 1988).

VCH treatment (7.5 mmol/kg/day × 10 days i.p.) caused a marked increase in CYP2A (3.7-fold), CYP2E1 (2.1-fold), and CYP2B (2.9-fold) immunoreactive protein levels (Fig. 4; Table 4). Only a marginal increase (1.3-fold) of CYP3A was produced by VCH treatment (Fig. 4; Table 4). Induction of CYP2A and CYP2B and not CYP3A in hepatic microsomes of VCH-treated mice is consistent with the steroid hydroxylase data (Figs. 2 and 3). The enhanced epoxidation of 1,2-VCHE observed in microsomes from DEX-pretreated mice (Table 2) is most likely due to elevated levels of CYP2B. Although microsomal P-450 levels were induced by VCH treatment, this treatment did not alter P-450 reductase activity (data not shown).

**Effect of VCH Treatment on Cytosolic GST Activity.** In addition to elevated microsomal P-450 activities, an increase in GST activity was observed in hepatic cytosol prepared from mice treated with VCH (7.5 mmol/kg/day i.p.; Table 5). A 1.5-fold increase in GST activity was observed after 5 days of VCH treatment, with a greater increase in activity at 10 and 15 days (Table 5).

**Effect of VCH Exposure on Plasma Levels of 1,2-VCHE and VCD.** Preliminary studies were conducted to measure circulating levels of the epoxides of VCH after administration of the parent compound. VCH (7.5 mmol/kg/day i.p.) was administered for either 5, 10, or 15 days; and 24 h later a challenge dose of VCH (7.5 mmol/kg i.p.) was administered. One hour after administration of the challenge dose, blood levels of the VCH epoxides were determined. Control animals received sesame seed oil for an equivalent number of days and then were challenged with a single dose of VCH.
1,2-VCHE blood levels were elevated after administration of VCH for 5 (p = .004) or 10 (p = .006) days as compared to controls (Table 6). Blood levels of VCD were significantly elevated at day 10 (p = .01, Table 6). Circulating levels of 1,2-VCHE and VCD were not elevated in animals pretreated for 15 days with VCH as compared to controls (Table 6).

To determine whether elevated VCH epoxide blood levels were due to an accumulation of these metabolites or due to VCH induction, a group of animals received VCH (7.5 mmol/kg/day i.p.) for 5, 10, or 15 days but did not receive a challenge dose 1 h before blood collection. VCH and its epoxides were not detected in the blood of these animals 24 h after the last dose (data not shown).

**Discussion**

The results of the present study indicate that repetitive doses of VCH in mice result in induction of hepatic P-450 and P-450-dependent activities. VCH exposure increased total P-450 content (35–83% above control levels) after either 5, 10, or 15 days of treatment. Elevated levels of P-450 were associated with increased expression of several isoforms, including 2E1 and members of the 2A and 2B subfamilies. In addition, elevated levels of these P-450 isoforms (CYP2A and CYP2B) correlated with marker androstenedione and testosterone hydroxylase activities. VCH was also capable of inducing its own metabolism. Microsomes prepared from mice pretreated with
Western blot analysis (Fig 4) using microsomes from B6C3F1 mice treated with VCH (7.5 mmol/kg/day x 10 days i.p.) or various inducing agents were prepared as described in Materials and Methods. Densitometry was performed and results have been normalized to control (i.e., nontreated mice).

Table 4

**Densitometry of Western blotting experiments**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fold of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2A</td>
</tr>
<tr>
<td></td>
<td>2B&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>2E&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>3A</td>
</tr>
<tr>
<td>None</td>
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</tr>
<tr>
<td>VCH</td>
<td>3.70</td>
</tr>
<tr>
<td>ACE</td>
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</tr>
<tr>
<td>PB</td>
<td>4.20</td>
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<tr>
<td>DEX</td>
<td>1.70</td>
</tr>
</tbody>
</table>

<sup>a</sup> Values listed for CYP2B-immunoreactive protein levels do not correspond to the immunoblot in Fig. 4. The data were obtained from a blot in which 10 μg of microsomal protein was loaded ( blot not shown), thus allowing for a immunoreactive band to be detected in microsomal fractions isolated from nontreated mice probed with rat CYP2B1.

Table 5

**GST activity in hepatic cytosol from VCH-treated B6C3F1 mice**

Hepatic cytosol was isolated from mice administered VCH or sesame oil for varying days of treatment with a subsequent challenge dose of VCH at 24 h, as outlined in Materials and Methods.

<table>
<thead>
<tr>
<th>Treatment</th>
<th>5 days</th>
<th>10 days</th>
<th>15 days</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sesame oil</td>
<td>1.40 (1.20–1.60)&lt;sup&gt;a&lt;/sup&gt;</td>
<td>1.37 (1.33–1.43)</td>
<td>1.33 (1.22–1.42)</td>
</tr>
<tr>
<td>VCH</td>
<td>2.14</td>
<td>2.72 (2.65–2.79)</td>
<td>2.75 (2.63–2.86)</td>
</tr>
<tr>
<td>(153%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(199%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>(207%)&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent the mean of duplicate determinations from two animals with the range of the individual values listed in parentheses, except for VCH 5 days, which is the mean of duplicate determinations from one animal.

<sup>b</sup> Numbers in parentheses represent the percentage of sesame oil control values.

Table 6

**Circulating levels of 1,2-VCHE and VCD in the blood of female B6C3F1 mice after administration of a single or multiple doses of VCH**

Animals received VCH (7.5 mmol/kg/day i.p.) or sesame oil (vehicle) for either 5, 10, or 15 days. On day 6, 11, or 16 a challenge dose of VCH (7.5 mmol/kg i.p.) was administered to both groups. Animals were killed 1 h after the challenge dose and epoxide blood levels determined.

<table>
<thead>
<tr>
<th>Epoxide</th>
<th>Treatment Days</th>
<th>Sesame oil</th>
<th>VCH</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,2-VCHE</td>
<td>5</td>
<td>20.00 ± 3.91</td>
<td>39.74 ± 6.52&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>26.11 ± 3.46</td>
<td>41.52 ± 8.21&lt;sup&gt;c&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>25.03 ± 5.25</td>
<td>24.38 ± 3.51</td>
</tr>
<tr>
<td>VCD</td>
<td>5</td>
<td>2.48 ± 0.81</td>
<td>3.72 ± 1.16</td>
</tr>
<tr>
<td></td>
<td>10</td>
<td>3.15 ± 0.44</td>
<td>5.13 ± 1.32&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>2.55 ± 0.56</td>
<td>3.08 ± 0.28</td>
</tr>
</tbody>
</table>

<sup>a</sup> Data represent the mean ± S.D. (N = 5, exception N = 4 for 1,2-VCHE levels reported for 10-day VCH treatment group).

<sup>b</sup> Value significantly different from control at p < 0.05.

<sup>c</sup> Value significantly different from control at p < 0.01.

VCH for 10 days demonstrated an increase (≥2-fold) in the rate of formation of the epoxides of VCH.

The induction of hepatic CYP2A and CYP2B by VCH treatment has important toxicological implications. Previous studies demonstrated that CYP2A and CYP2B are the principal P-450 isozymes involved in the biotransformation of VCH to 1,2-VCHE, accounting for 80% of epoxidation of VCH in untreated female mice (Smith et al., 1990c). Studies reported here demonstrated increased rates of epoxidation of 1,2-VCHE to VCD in hepatic microsomes isolated from mice pretreated with either VCH (2.1-fold) or phenobarbital (3.6-fold). Microsomal expression of CYP2A and CYP2B was also increased after VCH or PB treatment. Thus, preliminary data suggest that these P-450 isozymes may also play a role in the metabolism of 1,2-VCHE to VCD.

In addition, the data suggest partial involvement of CYP2E1 in the microsomal epoxidation of 1,2-VCHE to VCD. ACE, an inducer of CYP2E1 (Freeman et al., 1992), increased the microsomal rate of 1,2-VCHE epoxidation by 2-fold. Microsomal levels of this P-450 isoform were also elevated in VCH-treated mice. CYP2E1 has been shown to play a role in the metabolism of numerous low-molecular-weight organic compounds (Guengerich et al., 1991). In particular, the ovarian toxicant 1,3-butadiene is a substrate for CYP2E1 as well as its epoxide intermediate butadiene monooxepoxide (Csanyi et al., 1992; Duescher and Elfarra, 1994; Seaton et al., 1995). In addition, CYP2E1 is the principle enzyme involved in the epoxidation of styrene, a structural analog of VCH, to styrene 7,8-oxide. Similar to VCH, induction of styrene metabolism has been observed, with CYP2E1 being the major isozyme induced by styrene treatment (Elovaara et al., 1991). The specific role of these P-450 isozymes in the epoxidation of VCH requires further investigation.

An important finding of this work is the demonstration that VCD is formed in vivo after the administration of a single dose of VCH to mice. Although the metabolism of VCH has been well characterized in vitro (Smith et al., 1990a; Keller et al., 1997; Gervasi et al., 1980), this is the first evidence of VCD formation from VCH in vivo. Formation of the dipoxide was observed at a dose of VCH that, after repeated dosing, is known to deplete 85% of the small (primordial) follicles of mouse ovaries (Smith et al., 1990b; Hooser et al., 1994; Doerr et al., 1995). This is an important observation, because VCD has been shown to mediate VCH-induced ovarian toxicity (Doerr et al., 1995).

Previous work has established that consecutive daily doses of VCH are required for depletion of ovarian follicles (Smith et al., 1990b). The enhanced epoxidation of VCH and 1,2-VCHE observed in vitro after consecutive treatment of mice with VCH in vivo raised the intriguing possibility that induction of VCH epoxidation may play a critical role in the ovotoxicity of the compound. Therefore, preliminary in vivo studies were conducted to begin to assess the effect of repetitive dosing of VCH on circulating levels of VCH epoxides. Both 1,2-VCHE and VCD blood levels were significantly elevated after repetitive doses of VCH compared with animals receiving a single dose of VCH. The elevated epoxide levels after a challenge dose of VCH to VCH-pretreated animals were not due to accumulation of the epoxides during the pretreatment.

The significance of VCH induction, however, as it relates to the ovotoxic effects of this compound remains to be determined. In particular, it must be recognized that VCH may induce its own detoxification as well as bioactivation. Our data indicate an increase in cytosolic GST activity after repeated administration of VCH. 1,2-VCHE and VCD have been shown to be substrates for mouse GST (Boyland and Williams, 1965; Giannarini et al., 1981), and depletion of liver GSH after administration of either VCH, 1,2-VCHE, or VCD has been reported (Giannarini et al., 1981), suggesting the epoxides of VCH form GSH conjugates. The epoxides of VCH have also been shown to be substrates for epoxide hydrolase (Keller et al., 1997), another detoxification enzyme that was significantly induced after two doses of 1,2-VCHE (Giannarini et al., 1981). Therefore, changes in the rate of epoxide hydrolysis and conjugation with GSH after repeated exposure to VCH may attenuate enhanced levels of VCH epoxides due to VCH induction. This seems plausible based on the observation that circulating levels of 1,2-VCHE and VCD were not elevated in mice at day 15 of VCH treatment, although in vitro data...
at this time point demonstrated elevated levels of total P-450 and P-450-mediated activities.

Thus, repeated exposure to VCH results in induction of hepatic P-450 and P-450-mediated activities, including the in vitro epoxidation of VCH. Induction of select P-450 isozymes, including CYP2A and CYP2B, further supports their involvement in the metabolism of VCH. Whether induction of VCH metabolism plays a role in VCH-induced ovarian toxicity in mice remains to be determined. This current study demonstrates that mice are exposed to circulating levels of VCD, the ultimate ovotoxicant; however, a toxicokinetic study would be required to assess exposure to the epoxides after repeated administration of VCH.

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References


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