Expression and Activity of Cytochromes P450 2E1, 2A, and 2B in the Mouse Ovary: The Effect of 4-Vinylcyclohexene and Its Diepoxide Metabolite

Ellen A. Cannady,* Cheryl A. Dyer,† Patricia J. Christian,‡ I. Glenn Sipes,* and Patricia B. Hoyer‡

*University of Arizona, Department of Pharmacology and Toxicology, Tucson, Arizona 85721; †Northern Arizona University, Department of Biological Sciences, Flagstaff, Arizona 86011; and ‡University of Arizona, Department of Physiology, Tucson, Arizona 85724

Received November 21, 2002; accepted February 27, 2003

4-Vinylcyclohexene (VCH), an occupational chemical, causes destruction of small preantral follicles (F1) in mice. Previous studies suggested that VCH is bioactivated via cytochromes P450 (CYP450) to the ovotoxic, diepoxide metabolite, VCD. Whereas hepatic CYP450 isoforms 2E1, 2A, and 2B can metabolize VCH, the role of ovarian metabolism is unknown. This study investigated expression of these isoforms in isolated ovarian fractions (F1, 25–100 μm; F2, 100–250 μm; F3, >250 μm; interstitial cells, Int) from B6C3F1 mice dosed daily (15 days; ip) with vehicle, VCH (7.4 mmol/kg/day) or VCD (0.57 mmol/kg/day). Ovaries were removed and either isolated into specific ovarian compartments to chemical analysis, fixed for immunohistochemistry, or prepared for enzymatic assays. mRNA and protein for all isoforms were expressed/distributed in all ovarian fractions from vehicle-treated mice. In the targeted F1 follicles, VCH or VCD dosing increased (p < 0.05) mRNA encoding CYP2E1 (645 ± 14% VCH; 582 ± 16% VCD), CYP2A (689 ± 8% VCH; 730 ± 22% VCD), and CYP2B (246 ± 7% VCH) above control. VCH dosing altered (p < 0.05) mRNA encoding CYP2E1 in nontargeted F3 follicles (168 ± 7%) and CYP2A in Int (207 ± 19%) above control. Immunohistochemical analysis revealed the greatest staining intensity for all CYP isoforms in the Int. VCH dosing altered (p < 0.05) staining intensity in Int for CYP2E1 (19 ± 2.4% below control) and CYP2A (39 ± 5% above control). Staining intensity for CYP2B was increased (p < 0.05) above control in granulosa cells of small preantral (187 ± 42%) and antral (63 ± 8%) follicles. Catalytic assays in ovarian homogenates revealed that CYP2E1 and CYP2B were functional. Only CYP2E1 activity was increased (149 ± 12% above control; p < 0.05) by VCH dosing. The results demonstrate that mRNA and protein for CYP isoforms known to bioactivate VCH are expressed in the mouse ovary and are modulated by in vivo exposure to VCH and VCD. Interestingly, there is high expression of these isoforms in the Int. Thus, the ovary may contribute to ovotoxicity by promoting bioactivation of VCH to the toxic metabolite, VCD.

Key Words: cytochrome P450; 4-vinylcyclohexene; ovary; ovarian follicles; mouse; confocal microscopy.
Hepatic CYP2E1 was not enhanced in mice or rats following in vivo dosing with VCH. In hepatic microsomal incubations from CYP2E1-deficient and wild-type mice, VCH could be metabolized to similar amounts of VCH 1,2-monoepoxide and VCH 7,8-monoepoxide, suggesting that CYP2E1 is not required for epoxidation of VCH in the liver.

As regards to metabolism in extrahepatic tissues, the ovary may be an important tissue-specific site of bioactivation or detoxification. For example, Mattison et al. (1979) directly evaluated the role of ovarian metabolism of benzo(a)pyrene (B(a)P) in mice. $^3$H-B(a)P was incubated in vitro with the S9 fraction isolated from ovaries collected from mice dosed in vivo with vehicle control or 3-methylcholanthrene (3-MC). Numerous B(a)P metabolites were detected and greater amounts of product were formed in the tissue from 3-MC-treated animals. Studies by Shiromizu and Mattison (1984) evaluated the effect of unilateral intraovarian (i.o.) injection of B(a)P on the number of primordial oocytes in mice. B(a)P, which must be bioactivated to the toxic metabolite, was only ovotoxic in those ovaries injected with B(a)P, as evidenced by oocyte destruction. Additionally, oocyte loss was not as great when the potent CYP450 inhibitor, α-naphthoflavone, was administered (i.p.) concurrently with B(a)P (i.o.). Additional studies by Bengtsson et al. (1983, 1987, 1992) have also demonstrated ovarian metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) and 3-MC in rats by granulosa cells. These studies support the hypothesis that the ovary contains CYP450-dependent monooxygenases that can metabolize xenobiotics.

Although circulating levels of VCH and its metabolites can reach the ovary via the circulation, it is not known whether bioactivation of VCH or the monooxepoxides to VCD occurs in the ovary. Studies by Keller et al. (1997) showed that ovarian microsomes obtained from mice do not convert VCH to detectable levels of the epoxide metabolites in vitro, as analyzed by gas chromatography. However, previous in vivo exposure of mice to VCH may induce such a capacity. Furthermore, the possible compartmentalization of CYP450 enzymes/isoforms within different sizes of ovarian follicles has not been reported. Studies in our laboratory have demonstrated that the ovary has the capacity to be involved in detoxification reactions in both mice and rats. In mice, VCH or VCD dosing altered expression of mRNA and distribution of total protein, as well as functional activity for mEH in different sizes of ovarian follicles (Cannady et al., 2002). In rats, Flaws et al. (1994b) showed that isolated preantral follicles can detoxify VCD to the nontoxic tetroxetetral metabolite [4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane]. Thus, a number of studies have provided evidence to support the idea that extrahepatic metabolism within the ovary may directly amplify or attenuate the extent of ovarian toxicity caused by xenobiotic chemicals.

Because VCD specifically destroys small preantral (primordial and primary) follicles, ovarian distribution of CYP450 may impact susceptibility to exposure within specific follicle populations. Therefore, this study was designed to investigate ovarian CYP450 isoforms (CYP2E1, CYP2A, and CYP2B) by (1) assessing expression of mRNA in follicles isolated from mouse ovaries, (2) identifying ovarian distribution of protein, (3) measuring catalytic activity in whole ovaries, and (4) determining the effects of in vivo dosing with VCH and VCD on these CYP450 isoforms.

**MATERIALS AND METHODS**

**Reagents.** Medium 199 (M199) and custom-designed primers were purchased from In Vitrogen (Grand Island, NY). VCH (racemic mixture; purity 95–99%), dichloromethane, and 7-hydroxycoumarin were obtained from Aldrich Chemical Co. (Milwaukee, WI). VCD (mixture of isomers, composition unknown; purity >99%), collagenase (Clostridium histolyticum type I), deoxyribonuclease type I (DNase; from bovine pancreas), bovine serum albumin (BSA), sesame oil, N-(2-hydroxymethyl)pyrrolidin-N′-(2-ethanesulfonic acid) (HEPES), NaCl, ethylenediaminetetraacetic acid (EDTA), Tris, triton X, glyc erol, sodium dodecyl sulfate (SDS), NaF, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, potassium phosphate, MgCl₂, NADPH, coumarin, p-nitrophenol, p-nitroacetol, hydrochloric acid, trichloroacetic acid, sodium borate, and ribonuclease A were purchased from Sigma Chemical Co. (St. Louis, MO). 18S ribosomal RNA (18S rRNA) primers and RNAqueous kit were from Ambion Inc (Austin, TX). Reverse transcription system was from Promega (Madison, WI). Deoxynucleoside triphosphate (dNTP) mix, MgCl₂, and enzyme diluent were purchased from Idaho Technologies (Salt Lake City, UT). Advantage Plus taq polymerase was from Clontech Laboratories, Inc. (Palo Alto, CA). SYBR Green dye, 7-hydroxy-4-trifluoromethyl coumarin (HFC), 7-ethoxy-4-trifluoromethyl coumarin (EFC), and YOYO-1 were purchased from Molecular Probes (Eugene, OR). The bicinechonic acid (BCA) protein assay kit was obtained from Pierce (Rockford, IL). CYP2A6 antibody (rabbit anti-human) was purchased from Affinity BioReagents, Inc. (Golden, CO). CYP2B1 antibody (goat anti-rat) and CYP2E1 antibody (goat anti-rat) were purchased from Gentest (Woburn, MA). Rabbit anti-goat secondary antibody (CYP2E1, CYP2B1), goat anti-rabbit (CYP2A6) secondary antibody, and CY-5-streptavidin were obtained from Vector (Burlingame, CA).

**Animals.** Female B6C3F1 mice (day 21, d21) were purchased from Harlan Laboratories (Indianapolis, IN) and maintained in the University of Arizona Animal Care Facility. Animals (acclimated for 1 week prior to study) were housed in plastic cages, 5 animals per cage, and maintained in a controlled environment (22 ± 2°C, 12 h light/12 h dark cycles). The animals were provided a standard diet with ad libitum access to food and water. All animal experiments were approved by the University of Arizona’s Institutional Animal Care and Use Committee.

**Animal dosing.** Female B6C3F1 mice (day 28, d28; 10 animals/treatment group) were weighed and administered (intrapitoneal; ip) 15 daily, consecutive doses (2.5 μg/kg of body weight; 30–50 μl/dose) of either sesame oil (vehicle) or sesame oil containing VCH (7.4 mmol/kg/day) or VCD (0.57 mmol/kg/day). The equitoxic doses, routes of administration, and dosing time courses were based on previous studies performed in our laboratory (Smith et al., 1990b). Animals were killed by CO₂ inhalation 4 h following the final dose.

**Follicle isolation.** Ovaries were removed and the oviduct and excess fat were trimmed away. Ovaries were pooled (20 ovaries/treatment group), minced, and gently dissociated (40°C, 20 min) in Medium 199 (containing Hank’s salts, L-glutamine, 25 mM HEPES) with collagenase (7.5 mg/ml), DNase (0.267 mg/ml), and BSA (40 mg/ml). The dissociated mixture was filtered through a 250-mm screen via vacuum suction to exclude antral follicles (>250 μm; F3). The filtered follicles containing small (25–100 μm; fraction 1; F1) and large (100–250 μm; fraction 2; F2) preantral follicles and interstitial cells (int) were further hand sorted into distinct populations, using calibrated Pasteur pipettes (Flaws et al., 1994a). Ovarian fractions were stored (~80°C) until further use. Because F1 and F2 follicles are hand sorted during the
procedure, these follicular layers are relatively pure. The F3 follicles and Int cells are less pure, since these fractions may be contaminated by clumps of tissue that are not completely dissociated (F3) or by follicles that are extensively dissociated resulting in the loss of individual granulosa and theca cells (Int). However, the percent contamination is small and should not account for statistically significant differences between ovarian fractions.

RNA isolation. Total RNA was extracted from isolated ovarian fractions (20 pooled ovaries/treatment group) utilizing the RNeAqueous® kit protocol. Briefly, the samples were lysed and homogenized. The resulting mixture was applied to a filter cartridge, allowing the RNA to bind to the filter. After centrifugation, RNA was eluted from the filter, and its concentration was determined via UV spectrometry (λ = 260/280 nm; Beckman DU-64).

First-strand cDNA synthesis. Total RNA (0.75 μg) was reverse transcribed into cDNA utilizing the Reverse Transcription System®. Following reverse transcription utilizing random primers, the resulting cdNA was precipitated (ethanol, −80°C overnight). The excess supernatant was removed, and the pellet was resuspended in PCR-grade water (100 μl).

Real-time polymerase chain reaction. cDNA (1 μl) from various ovarian fractions was used to perform relative, semi-quantitative PCR utilizing a LightCycler® (Idaho Technology) capable of real-time PCR. The LightCycler® quantifies the amount of PCR product generated by measuring the dye (SYBR green), which fluoresces when bound to double stranded DNA. Custom designed primers were utilized (CYP2E1; forward primer: 5'-GTC TTT AAC CAA GTT GGC AA 3'; reverse primer: 5'-CCA ATC AGA AAG GTA GGG TC 3' [Freeman et al., 1992]; CYP2A; forward primer: 5'-ATT GCC ACC ACC TTC TAC CT 3'; reverse primer: 5'-CAG TAT TGG GGT TCT TCT CTC CC 3' [Marc et al., 1999]). Amplification conditions for CYP2E1 were 95°C/0 s (denaturing), 57°C/0 s (annealing), and 72°C/10 s (extending) for 50 cycles. Amplification conditions for CYP2A were 95°C/0 s (denaturing), 68°C/0 s (annealing), and 72°C/12 s (extending) for 50 cycles. Amplification conditions for CYP2B were 95°C/0 s (denaturing), 64°C/0 s (annealing), and 72°C/16 s (extending) for 50 cycles. A standard curve was generated from 1:5 dilutions of each RNA. Each dilution was used as PCR template to generate a standard curve.

Liver microsome preparation. Following euthanization, livers were excised from B6.CF1 mice. Microsomes were prepared according to Guengerich (1989). Briefly, livers were homogenized in buffer (pH 7.4) containing 1 M Tris–HCl, 1 M KCl, 100 mM EDTA, and 20 mM BHT. Microsomes were isolated by differential ultracentrifugation. The microsomal fraction was collected and the protein concentration was determined utilizing the BCA Protein Assay kit.

CYP450 functional assays. Specific activities for CYP2E1, CYP2A, and CYP2B were evaluated in whole ovarian homogenates utilizing model substrates. CYP2E1 catalyzes the hydroxylation of p-nitrophenol to p-nitrocatechol (Patten et al., 1992), while CYP2A catalyzes the hydroxylation of coumarin to 7-hydroxycoumarin (Waxman et al., 1991), and at low concentrations of substrate (~5 μM), CYP2B specifically catalyzes the metabolism (7-O-deethylation) of 7-ethoxy-4-trifluoromethyl coumarin (EFC) to 7-hydroxy-4-trifluoromethyl coumarin (HFC; Code et al., 1997). Protein (200 μg) from whole ovarian homogenates was incubated (37°C, shaking water bath) in assay buffer containing substrate and cofactor according to conditions for specific assays listed in Table 1. Following incubation, the CYP2E1 reaction was stopped by adding 20% trichloroacetic acid to samples, and samples were placed on ice. The samples were extracted with dichloromethane and concentrated (~50 μl), and the supernatant was transferred to a 96-well plate. Immediately prior to reading, 2-M NaOH was added to each sample or standard. In the CYP2A activity assay, 2-N hydrochloric acid was added to stop the reaction, and samples were placed on ice. The samples were extracted with dichloromethane and concentrated (~50 μl), back extracted with sodium borate (30 mM), and the aqueous layer was transferred to a 96-well plate. In the CYP2B activity assay, 2-N hydrochloric acid was added to stop the reaction and samples were placed on ice. The samples were concentrated (~50 μl), and the supernatant was transferred to a 96-well plate. Immediately prior to reading, 0.1-M Tris (pH 9.0) was added to each sample or standard. In the CYP2E1 assay, absorbance was measured at λ = 520 nm on a 96-well plate reader (Molecular Devices) equipped with Soft Max Pro Software. Fluorescence for the CYP2A and CYP2B activity assays (excitation λ = 410 nm; emission λ = 510 nm) was measured on a fluorescent plate reader (Spectra Max Gemini XR) equipped with Soft Max Pro Software. In all CYP450 functional assays, the amount of product formed (expressed as nmol product formed/min/mg protein) in each unknown sample was determined by linear regression analysis. Additionally, experiments were performed to ensure linearity of the reaction in terms of incubation time (15 min–2 h) and protein concentration (50–1000 μg). To verify assay measurements, the experiments were also performed in liver microsomes (data not shown). Optimal conditions for p-nitrophenol hydroxylation (CYP2E1) and EFC metabolism (CYP2B) in whole ovaries utilized 200 μg of protein incubated for 1 h.

Statistical analyses. Comparisons between treatment groups and ovarian compartments were made using one-way analysis of variance (ANOVA). When significant differences were detected, individual groups were compared.
with the Fisher protected least significant difference (PLSD) multiple range test. Significance was assigned at $p < 0.05$.

**RESULTS**

**mRNA Expression**

mRNA encoding various CYPP450 isoforms was evaluated in follicles isolated from mouse ovaries following repeated, *in vivo* exposure (15 daily doses) to vehicle control. mRNA encoding CYP2E1, CYP2A, and CYP2B was detected in all ovarian fractions that were studied (F1, small preantral, 25–100 μm; F2, large preantral, 100–250 μm; F3, antral, >250 μm; Int) from all treatment groups. Relative expression levels for mRNA encoding the CYP450 isoforms in each ovarian fraction from vehicle-treated animals are shown in Table 2. CYP2E1 was highly expressed in F2 and F3 follicles. CYP2A and CYP2B were expressed significantly more ($p < 0.05$) in Int cells, compared to CYP2E1, as well as the other ovarian fractions. As shown in Figure 1, repeated daily dosing (15 days) with VCH or VCD increased ($p < 0.05$) mRNA encoding CYP2E1 in F1 follicles (645 ± 14% above control, VCH; 582 ± 16% above control, VCD) and F3 follicles (168 ± 7% above control, VCH), compared to control. There was little effect of dosing in the other ovarian fractions (Fig. 1A). mRNA encoding CYP2A was increased ($p < 0.05$) in F1 follicles (689 ± 8% above control, VCH; 730 ± 22% above control, VCD) and Int cells (207 ± 19% above control, VCH), but dosing had little effect in F2 and F3 follicles (Fig. 1B). Follow­ing VCH dosing mRNA encoding CYP2B was increased (246 ± 7% above control; $p < 0.05$) in F1 follicles, but not in other fractions nor in any fraction following VCD treatment (Fig. 1C). In all, dosing with VCH increased expression of all CYP450 isoforms in the F1 follicles.

**Protein Expression**

Total protein for CYP2E1, CYP2A, and CYP2B, as visualized by immunostaining in ovarian sections, was observed in oocytes, granulosa cells, and theca cells, as well as the surrounding interstitial (Int) cells. Following repeated dosing, VCH and VCD affected distribution of CYP450 isoforms. CYP2E1 protein was reduced ($p < 0.05$) in Int cells (19 μg 2% below control, VCH), but remained comparable to controls in other compartments (Figs. 2A,B). VCH dosing caused an increase ($p < 0.05$) in staining intensity for CYP2A protein in the Int cells (39 μg 5% above control, VCH). No effects of VCH or VCD dosing on CYP2A were observed in other sizes of ovarian follicles (Figs. 2A,B). CYP2B staining intensity was increased ($p < 0.05$) in granulosa cells of small primary follicles (F1; 187 ± 42% above control) following dosing with

<table>
<thead>
<tr>
<th>TABLE 1</th>
<th>Conditions for Specific Activity Assays</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>200 μg</td>
</tr>
<tr>
<td>Buffer</td>
<td>100 mM potassium phosphate; pH 6.8</td>
</tr>
<tr>
<td>Cofactor (NADPH)</td>
<td>20 mM</td>
</tr>
<tr>
<td>Substrate</td>
<td>5 mM p-nitrophenol</td>
</tr>
<tr>
<td>Standards</td>
<td>p-nitrocatechol; 10 mM–40 μM</td>
</tr>
<tr>
<td>Incubation Volume</td>
<td>250 μL</td>
</tr>
<tr>
<td>Incubation Time</td>
<td>1 h</td>
</tr>
<tr>
<td>References</td>
<td>Chang et al., 1998b;</td>
</tr>
<tr>
<td></td>
<td>Tierney et al., 1992</td>
</tr>
</tbody>
</table>

$^a$ 7-ethoxy-4-trifluoromethyl coumarin (EFC).

$^b$ 7-hydroxy-4-trifluoromethyl coumarin (HFC).

### TABLE 2

Unstimulated Expression of mRNA Encoding CYP450 Isoforms in Ovarian Fractions$^{a,b}$

<table>
<thead>
<tr>
<th>CYP450 isoform</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>Int</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP2E1</td>
<td>0.005 ± 0.001</td>
<td>0.776 ± 0.55</td>
<td>0.774 ± 0.45</td>
<td>0.324 ± 0.16</td>
</tr>
<tr>
<td>CYP2A</td>
<td>0.027 ± 0.03</td>
<td>0.044 ± 0.02</td>
<td>0.680 ± 0.6</td>
<td>2.082 ± 0.6$^c$</td>
</tr>
<tr>
<td>CYP2B</td>
<td>0.133 ± 0.06</td>
<td>0.506 ± 0.43</td>
<td>0.600 ± 0.46</td>
<td>2.903 ± 0.12$^c$</td>
</tr>
</tbody>
</table>

$^a$ mRNA—arbitrary units, ratio of CYP isoform : 18S ribosomal RNA.

$^b$ Ovarian fractions obtained from vehicle control-treated mice.

$^c$ different ($p < 0.05$) from CYP2E1.
VCH. Increased staining intensity for CYP2B was also observed in granulosa cells of antral follicles (F3) following dosing (63 ± 8% above control, VCH; 45 ± 8% above control, VCD; Figs. 2E–G).

**Functional Activities**

Functional activities for CYP2E1, CYP2A, and CYP2B were analyzed in whole ovarian homogenates from mice following in vivo dosing (15 daily doses) with vehicle control, VCH, or VCD. Basal CYP2E1 activity in vehicle control mice was 0.0726 ± 0.015 nmol/min/mg (Fig. 3). CYP2A activity could not be detected. Basal CYP2B activity in vehicle-treated ovaries was 0.0037 ± 0.001 nmol/min/mg (Fig. 3). Repeated dosing with VCH enhanced (p < 0.05) CYP2E1 activity (0.181 ± 0.022 nmol/min/mg; Fig. 4), whereas dosing with VCD did not (0.126 ± 0.020 nmol/min/mg; Fig. 4). Dosing with either VCH or VCD did not affect CYP2B activity (0.0036 ± 0.001 nmol/min/mg, VCH; 0.0034 ± 0.001 nmol/min/mg, VCD; Fig. 5). No specific activity for CYP2A could be measured in ovarian homogenates from any treatment group, although linearity of hydroxylation of coumarin to 7-hydroxycoumarin was measured in liver microsomes. All functional assays were validated in hepatic microsomes from mice.

**DISCUSSION**

The results of these studies suggest that the ovary likely contributes to its own susceptibility to VCH/VCD ovotoxicity. First, the metabolizing enzymes involved in VCH metabolism are present in susceptible follicle populations and associated compartments. Second, ovarian CYP enzymes are induced after exposure, suggesting that the ovary responds to exposure.

Although the present study did not directly evaluate the ovarian metabolism of VCH, the results provide indirect support for an involvement of the ovary in VCH-induced ovotoxicity. For instance, repeated in vivo dosing with VCH or VCD altered CYP450 expression. A significant increase in expression of mRNA encoding CYP2E1 was observed in F1 follicles, those follicles specifically targeted by VCD, as well as non-targeted F3 follicles after VCH or VCD dosing. Although total protein for CYP2E1 was slightly decreased in Int cells following VCH-treatment, whole ovarian CYP2E1 enzyme activity was increased. Due to low levels of enzyme activity, the measurements were performed in whole ovarian homogenates, thus making it impossible to identify the specific ovarian compartment that is functionally responsible for metabolism of the model substrate. Based on the immunohistochemical data, any size of follicle could potentially convert the model substrate, p-nitrophenol. However, due to the abundant CYP2E1 protein distribution in the interstitium, this ovarian compartment would likely contribute most significantly to this metabolism.
A significant increase in expression of mRNA encoding CYP2A was observed in the targeted F1 follicles following VCH or VCD dosing. VCH dosing also increased expression of CYP2A in the Int cells. Staining intensity for CYP2A protein was significantly increased in the Int cells. Although this correlates with the mRNA data, the changes in mRNA levels and total protein do not correlate with functional CYP2A activity towards the model substrate, coumarin, which was not detectable in any treatment group. Specific activity for CYP2A towards other model substrates was not evaluated in the study design. Thus, it is not known whether a lack of detection of CYP2A activity was due to reduced sensitivity of the enzyme towards coumarin, or whether it was because CYP2A protein is expressed in the ovary but does not possess functional activity.

mRNA encoding CYP2B was increased in F1 follicles following repeated dosing with VCH. This was consistent with the VCH-induced increase in CYP2B protein in granulosa cells of small primary (F1) follicles. Furthermore, VCH and VCD dosing increased protein in F3 follicles. Although CYP2B activity was measured in whole ovarian homogenates, it was not significantly affected by VCH or VCD dosing. However, if there was a specific effect of dosing in F1 follicles, it might

---

**FIG. 2.** Ovarian distribution of CYP2E1, CYP2A, and CYP2B protein in ovine follicles and interstitial cells. B6C3F1 mice (day 28) were dosed daily (15 day; ip) with sesame oil (vehicle), VCH (7.4 mmol/kg/day), or VCD (0.57 mmol/kg/day). Ovaries were removed 4 h after the final dose and processed for immunostaining by confocal microscopy, as described in methods. Green stain = YOYO 1 (DNA stain). Red stain = CY-5 labeled antibody for each specific CYP450 isoform. CYP2E1 protein was visualized in (A) F1, F2, and Int from vehicle-treated mice, and (B) F1, F2, and Int from VCH-treated mice, in which the staining intensity in Int cells was significantly decreased compared to control ($p < 0.05$). CYP2A protein was visualized in (C) F1, F2, and Int from vehicle-treated mice, and (D) F1, F2, and Int from VCH-treated mice, in which the staining intensity in Int cells was significantly increased compared to control ($p < 0.05$). CYP2B protein was visualized in (E) F1, F2, and Int from vehicle-treated mice, (F) F1, F2, and Int from VCH-treated mice, in which the staining intensity in Int cells was significantly decreased compared to control ($p < 0.05$), and (G) F1, F2, and Int from VCH-treated mice, in which the staining intensity in granulosa cells from antral follicles was significantly increased compared to control ($p < 0.05$). (H) Ovarian section stained with YOYO-1 and CY-5, with no primary antibody added. All samples were normalized to control so multiple experiments could be compared. $n = 2$.

**FIG. 3.** Unstimulated CYP450 activity in whole ovaries. Ovaries were collected, and homogenates were prepared from vehicle-treated mice (day 42). Total protein was isolated from ovarian homogenates. Protein (200 μg) was incubated for 1 h with sample substrates as described in methods. The product was concentrated and absorbance (CYP2E1; $λ = 520$ nm) or fluorescence (CYP2A, CYP2B; $λ_{em} = 425$ nm, $λ_{em} = 525$ nm) was measured. Values were determined by linear regression and are expressed as nmol/min/mg of protein. Data represent mean ± SE ($n = 4–8$; * different from CYP2B, $p < 0.05$; ND = not detectable).
have been masked by unchanged activities in larger ovarian compartments.

Our studies evaluated mRNA expression, total protein, and functional activity following 15 days of repeated daily dosing with VCH or VCD. The results suggest that CYP2E1 is a likely contributor to ovarian metabolism, since baseline activity toward a model substrate was significantly higher than that of CYP2A or CYP2B. This is even more likely because activity for CYP2E1 was significantly increased by repeated dosing with VCH for 15 days. However, the effect of dosing on CYP2E1, CYP2A, and CYP2B expression profiles in the ovary are not known at earlier or later time points. Interestingly, in previous studies CYP2E1 did not appear to be involved in the bioactivation of VCH in liver (Fontaine et al., 2001a,b). Rather, those studies showed induction of total and functional hepatic protein for CYP2A and CYP2B following 10 days of dosing with VCH in the mouse. Thus, different isoform profiles may predominate in the ovary as compared to the liver. Additionally, different dosing regimens may impact isoform expression patterns.

Our studies showed that mRNA encoding CYP2E1, CYP2A, and CYP2B is increased in the vulnerable population of F1 follicles following repeated dosing with VCH or VCD. However, other sizes of ovarian follicles and Int cells also express the metabolic enzymes and may contribute to providing the target population of follicles with the bioactive metabolite. Interestingly, there was a high level of staining intensity for all of the CYP450 isoforms localized in the Int compartment. Thus, this ovarian compartment may be involved in either mono-epoxidation of VCH and/or subsequent epoxidation of the monoepoxides to VCD. This hypothesis is plausible when one considers that the Int cells are in the highly vascularized region of the ovary (Erickson et al., 1985). Such cells would likely be exposed to toxicants traveling through the blood stream. On a broader scale, this metabolic potential may serve as a generalized function for the interstitium, whose physiological function has remained largely unclear. Because enzyme induction was observed in ovarian compartments not targeted by VCH or VCD, an interesting point to consider is the protection against ovotoxicity granted to the nontargeted populations, despite the presence and activity of bioactivating enzymes. These compartments also contain detoxifying enzymes, such as mEH. The balance between bioactivation and detoxification enzymes may favor detoxification in these compartments. For example, Flaws et al. (1994b) showed in rats that large preantral follicles (F2) converted more VCD to the inactive tetrol metabolite compared to small preantral follicles (F1). Cannady et al. (2002) showed in mice that baseline mEH activity was greater in F2 follicles compared to other sizes of follicles and Int cells. Additionally, the susceptibility of the F1 follicles compared to other compartments may be due to inherent differences in morphology (e.g., lack of theca cell layer) as well as physical properties of the chemical, thus governing the specificity of the response.

Although the relative contribution of ovarian metabolism, compared to hepatic metabolism, in VCH-induced ovotoxicity is not known, it has been demonstrated here that the ovary has the potential (mRNA and functional protein) for bioactivation of VCH and/or the monoepoxides. Based collectively on the basal and VCH-induced levels of CYP450 activity, CYP2E1 is the most likely isoform that would be involved in ovarian metabolism. Additionally, we conclude that the interstitial (Int) compartment is potentially the most important ovarian compartment involved in bioactivation of VCH, due to its high level of expression of all three of the CYP450 isoforms. Future studies will be aimed at determining more precisely the extent to which the ovary contributes to VCH-induced ovotoxicity in mice. This information can serve to better elucidate the meta-
bolic role of the ovary in responding to xenobiotic exposures, which may impact ovarian function in women.

ACKNOWLEDGMENTS

This work is supported in part by NIH grant ES08979 (PBH), ES09246 (PBH), NIEHS Center grant ES06694, and NIEHS Training grant ES07019.

REFERENCES


