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THE ROLE OF OVARIAN METABOLISM IN 4-VINYLCYCLOHEXENE-INDUCED OVOTOXICITY IN B6C3F1 MICE

by

Ellen Annette Hollis Cannady

A Dissertation Submitted to the Faculty of the COMMITTEE OF PHARMACOLOGY AND TOXICOLOGY (GRADUATE) In Partial Fulfillment of the Requirements For the degree of DOCTOR OF PHILOSOPHY In the Graduate College THE UNIVERSITY OF ARIZONA 2002
As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Ellen Annette Hollis Cannady entitled "The Role of Ovarian Metabolism in 4-Vinylcyclohexene-Induced Ovotoxicity in B6C3F1 Mice." and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy.

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Signed: Ellen J. Kennedy
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DEDICATION

This work is dedicated to my family and is in loving memory of my grandfather, J.C. Hollis, and my great aunt, Annette B. Cassidy. Their strong family and educational values have been instilled in me forever.
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<tr>
<td>B(a)P:</td>
<td>benzo(a)pyrene</td>
</tr>
<tr>
<td>BD:</td>
<td>1,3-butadiene</td>
</tr>
<tr>
<td>BDE:</td>
<td>butadiene dicpoxide</td>
</tr>
<tr>
<td>BMO:</td>
<td>butadiene monoepoxide</td>
</tr>
<tr>
<td>CHE:</td>
<td>cyclohexene oxide</td>
</tr>
<tr>
<td>CL:</td>
<td>corpus luteum</td>
</tr>
<tr>
<td>CSO:</td>
<td>cis-stilbene oxide</td>
</tr>
<tr>
<td>DMBA:</td>
<td>7,12-dimethylbenz(a)anthracene</td>
</tr>
<tr>
<td>EFC:</td>
<td>7-ethoxy-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>F1:</td>
<td>fraction 1 follicles</td>
</tr>
<tr>
<td>F2:</td>
<td>fraction 2 follicles</td>
</tr>
<tr>
<td>F3:</td>
<td>fraction 3 follicles</td>
</tr>
<tr>
<td>FSH:</td>
<td>follicle stimulating hormone</td>
</tr>
<tr>
<td>GC:</td>
<td>granulosa cell</td>
</tr>
<tr>
<td>GD:</td>
<td>gestational day</td>
</tr>
<tr>
<td>HFC:</td>
<td>7-hydroxy-4-trifluoromethyl coumarin</td>
</tr>
<tr>
<td>Int:</td>
<td>interstitial cells</td>
</tr>
<tr>
<td>LH:</td>
<td>luteinizing hormone</td>
</tr>
<tr>
<td>3-MC:</td>
<td>3-methylcholanthrene</td>
</tr>
<tr>
<td>mEH:</td>
<td>microsomal epoxide hydrolase</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
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<tr>
<td>PAH</td>
<td>polycyclic aromatic hydrocarbon</td>
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<tr>
<td>PND</td>
<td>postnatal day</td>
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<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<tr>
<td>Tetrolo</td>
<td>4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane</td>
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<tr>
<td>VCD</td>
<td>vinylcyclohexene diepoxide</td>
</tr>
<tr>
<td>VCH</td>
<td>vinylcyclohexene</td>
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<tr>
<td>1,2-VCM</td>
<td>vinylcyclohexene 1,2-monoepoxide</td>
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ABSTRACT

4-Vinylcyclohexene (VCH), an industrial chemical, causes destruction of small pre-antral follicles (F1) in mice. Previous studies suggested that VCH is heptatically bioactivated to its ovotoxic metabolite, vinylcyclohexene diepoxide (VCD), by the cytochrome P450 enzymes, likely Cyp 2E1, Cyp 2A, and Cyp 2B. Additionally, microsomal epoxide hydrolase (mEH) likely participates in detoxifying the epoxide metabolites. The role of ovarian metabolism (bioactivation/ detoxification) of VCH and its metabolites is not known. The hypothesis of this dissertation research was that ovarian metabolism contributes to VCH-induced ovotoxicity. These studies investigated whether the mouse ovary expresses a) mRNA, b) total protein, and c) functional protein for several metabolic enzymes (Cyp 2E1, Cyp 2A, Cyp 2B, mEH), as well as, d) the effects of VCH/VCD dosing on these parameters. Female B6C3F1 mice were dosed (i.p.) daily (15 d) with VCH (7.4 mmol/kg) or VCD (0.57 mmol/kg). Ovaries were removed and enzymatically digested and sorted into specific ovarian fractions (F1. small preantral; F2. large preantral; F3. antral; Int. interstitial cells) for mRNA analysis by realtime PCR, fixed for immunohistochemistry by confocal microscopy, or homogenized for functional assays. Basal expression was detected for mRNA encoding Cyp 2E1, Cyp 2A, Cyp 2B, and mEH in all ovarian fractions. In vivo dosing with VCH/VCD differentially altered expression, as expression increased for all enzymes in targeted F1 follicles. All enzymes were also distributed throughout the ovary, with high immunostaining intensity in the Int. In vivo dosing with VCH/VCD also affected protein distribution. Utilizing model
substrates, catalytic activity was evaluated in ovarian fractions or whole ovaries. Basal activity was detected for Cyp 2E1, Cyp 2B, and mEH, while VCH dosing only induced activities for Cyp 2E1 and mEH. Taken together, the ovary has the metabolic capacity to be involved in metabolic reactions. Bioactivation is likely via Cyp 2E1 in the Int. cells. Although the relative contribution of ovarian metabolism in VCH-induced ovotoxicity is not known, the ovary likely plays a greater role in detoxification, due to greater levels of mEH activity. Thus, the ovary may provide a metabolic contribution to mediating the effects of ovotoxicants.
CHAPTER 1

INTRODUCTION

Ovarian Follicular Development:

The mammalian ovary is a heterogeneous organ (Figure 1.1). It contains a variety of follicles in different stages of development. The smallest follicles, the primordial follicles, contain only an oocyte surrounded by a fusiform layer of granulosa cells. The follicle is surrounded by a basement membrane (Hirshfield, 1994). These follicles are essentially dormant, as the oocyte is arrested in prophase of the first meiotic division. As the primordial follicle is recruited to develop and mature, through an unknown mechanism, several changes take place. First, the oocyte dramatically increases in size from approximately 15 μm to 100 μm in diameter in the human. At this point, the oocyte is thought to be fully mature, and in the rat, has been shown to be physiologically capable of resuming meiotic maturation (Erickson, 1978). Secondly, the zona pellucida, which is made up of glycoproteins, forms around the outer edge of the oocyte, and the flattened, squamous granulosa cells of the primordial follicle differentiate to become more cuboidal in shape. This change in morphology is thought to initiate mitosis in some of the granulosa cells, an important step in follicle growth and development. The actively developing follicle gains multiple granulosa cells, resulting in formation of a primary follicle (Erickson, 1978). During continued growth and maturation of the primary follicle, the follicle acquires a number of receptors, notably follicle-stimulating
Figure 1.1: Mammalian Ovary. Diagram of mammalian ovary illustrating the different stages of follicular development. Distinct populations of follicles include Fraction 1 (F1; 25-100 μm in diameter), Fraction 2 (F2; 100-250 μm in diameter), and Fraction 3 (F3; >250 μm in diameter). The interstitium compartment makes up the remaining volume of the ovary. After the follicle is ovulated, the corpus luteum (CL) is formed.
hormone (FSH), estrogen, and testosterone. When the follicle approaches 150 μm in diameter, the follicle migrates from the avascular, ovarian cortex, to the vascular ovarian medulla. Additionally, the follicle acquires another cell type surrounding the granulosa cells, termed the theca interna, which consists of a capillary network supplied by one or two arterioles, in addition to a steroid secreting component (Erickson et al., 1985). The theca externa, a layer of cells made up of connective tissue, forms the outer layer of the follicle (Hirshfield, 1994). The follicle is now termed a secondary follicle. The secondary follicle continues to grow and develop under the direct influence of hormones. The granulosa cells proliferate in response to FSH stimulation and the presence of FSH induces the formation of luteinizing hormone (LH) receptors on the theca cell. The follicle also gains progesterone and prolactin receptors. A fluid filled antrum forms, which continues to grow in size as the follicle grows. The arteriole blood supply also undergoes anastomosis, becoming significantly more extensive as the follicles grows. It is thought that the pressure created by the fluid in the antrum, in addition to enzymatic proteolysis of the basement membrane, helps expel the oocyte at the time of ovulation. Following ovulation, the granulosa cells differentiate to form the corpus luteum, which functions to produce progesterone to help maintain pregnancy, if the oocyte is fertilized. If the oocyte is not fertilized, the corpus luteum degenerates. The interstitial cells are those cells that help support the ovary's structure. These cells are in the most vascular region of the ovary. There are four general types of interstitial cells, including primary interstitial cells, theca interstitial cells, secondary interstitial cells, and hilus interstitial cells. The primary interstitial cells develop in utero in the medulla region of the ovary.
In humans, the primary interstitial cells have a short life span, appearing at 12 weeks gestation and then disappearing by 20 weeks gestation. These cells are not functional. The theca interstitial cells are located in the spaces between the theca interna of developing follicles. The origin of these cells is unknown, however, it is postulated that they arise from unspecialized mesenchymal cells located near the developing secondary follicle. The theca interstitial cells are steroidogenic and produce androgens. The secondary interstitial cells are derived from the theca interna of atretic follicles. When a follicle dies, via an apoptotic-like process termed atresia, the oocyte and granulosa cells are cleared from the ovary by phagocytic cells (Benson, 1998). However, the theca interna survive, undergo hypertrophy, and settle into the region of the old follicle. These cells become the secondary interstitial cells. In addition, they continue to respond to LH and synthesize androgens. The hilus interstitial cells reside along the length of the ovarian hilus. These cells can proliferate and differentiate into testosterone secreting cells. Despite this function, however, the physiological importance of these cells is unclear (Erickson et al., 1985; Hirshfield, 1994). Furthermore, other than the synthesis and secretion of steroid hormones (most notably androstenedione and testosterone), the function of the theca interstitial cells, the secondary interstitial cells, and the hilus interstitial cells these cells, has largely remained unclear.

*In utero*, a human female ovary contains approximately 6 million primordial germ cells. At birth, this number is greatly reduced to about 2 million. After the first few days of post-natal life, the oocytes become surrounded by somatic follicular cells to become primordial follicles. By puberty, the number of primordial follicles is further reduced to
approximately 400,000. Only about 400 oocytes will be ovulated in a female's lifetime. Therefore, not every single follicle will be recruited to develop and not every developing follicle will complete the maturation process and be ovulated. In fact, over 99.9% of the follicles will undergo a natural form of cell death, termed either attrition, which occurs during fetal development, or atresia. Although it is not known what triggers atresia in a follicle, the mechanism for atresia is thought to be via a hormonally-controlled apoptotic-like pathway. Kaipia and Hsueh (1997) noted that “sufficient exposure of antral follicles to FSH is the most critical stimulus for the follicles to escape atresia and reach the preovulatory follicle stage.” Thus, there appears to be a balance between so called “survival factors” and “atretogenic factors.” Survival factors are thought to include the gonadotropins FSH and LH, which suppress follicular apoptosis by augmenting local factors such as interleukin-1β (IL-1β), estrogen, and insulin-like growth factor-1 (IGF-1). IL-1β increases the synthesis of nitric oxide, which activates the cGMP-dependent pathway and inhibits apoptosis. Estrogen is required for growth and maturation of follicles. Studies have shown that atretic follicles produce less estrogen, suggesting that an appropriate estrogen/androgen ratio is required to maintain healthy follicles. Thus, the presence of estrogen would inhibit follicular atresia. Finally, IGF-1 is thought to act with gonadotropins to promote differentiation of granulosa cells, thus suppressing follicle apoptosis (Chun et al., 1994). On the reverse side, atretogenic factors include tumor necrosis factor-α (TNF-α), Fas, interleukin-6 (IL-6), and androgens. Both the TNF-α and Fas receptors contain death domains. “When early antral follicles are cultured in the presence of FSH, a dose-dependent increase in apoptosis can be seen with increasing
concentrations of TNF-α," wrote Kaipia and Chun (1996). When human granulosa/luteal cells are cultured in the presence of Fas, apoptosis is also induced (Quirk et al., 1995). Furthermore, production of the cytokine, IL-6, by the granulosa cells has been shown to cause DNA fragmentation, another measure of apoptosis. Finally, increased levels of androgens are thought to promote atresia by altering the estrogen:androgen ratio, thus decreasing the amounts of the protective estrogens and increasing the amounts of androgen.

Although the triggers of atresia at various stages of follicle development are not completely understood, the ultimate result is ovarian follicle loss. This is important because the ovary contains a finite number of follicles, which once destroyed cannot be regenerated. Thus, as follicles are depleted, the volume of secondary interstitium increases. Furthermore, when the ovary is completely depleted of follicles, at menopause, it is not surprising that only the interstitium remains.

**Ovotoxic Chemicals and their Consequences on Reproduction:**

Understanding the concept that ovarian follicles cannot be regenerated becomes important when one considers toxic agents and drugs that can cause follicle loss. Depending on the follicle population targeted by such agents, this can result in a range of effects from temporary infertility to premature ovarian failure. Thus, different targeted populations of follicles have different outcomes. For instance, if a pre-pubertal female is exposed to a toxicant that depletes primordial follicles, sterility could result, or she could have a shortened reproductive lifespan. If instead, the young female is exposed to a
toxicant that targets antral follicles, theoretically no damage should ensue, since follicles do not develop to this stage of maturity until after the onset of puberty. If an adult female is exposed to a toxicant that targets antral follicles, temporary infertility will result. The female will stop cycling. Once the toxicant is removed, cyclicity will resume because there is still a pool of primordial follicles from which follicles can be recruited to develop, mature, and be ovulated. However, if a toxicant targets the primordial follicles, the female will continue cycling because the large follicle pool is not yet affected. The female will eventually stop cycling when there is no longer a pool of primordial follicles from which to recruit. This type of exposure results in premature ovarian failure, or menopause in the human. Unfortunately, this is referred to as a “silent toxicity.” since in most cases the female does not know that she has been exposed to a toxicant until it is too late and the damage has already occurred. If a pregnant female is exposed to ovotoxic chemicals or drugs, the pregnancy could fail, there could be lactational consequences, or the agent could be passed through the placenta and affect the fetus in utero. Again, if the agent targets and destroys primordial follicles, the female offspring would be sterile. At the opposite end of the reproductive lifespan, exposure to ovotoxicants in post-menopausal women might result in the increased incidence of ovarian neoplasms (Hoyer, 1997).

A variety of agents including ionizing radiation, drugs, and environmental contaminants have been shown to cause ovarian toxicity (Mattison and Shulman, 1980). Ionizing radiation destroys oocytes in both rodents and humans by destroying chromatin. The chromatin structure is more diffuse in small oocytes, while it is denser in larger
oocytes, which is thought to make the chromosomes more resistant to the effects of ionizing radiation. Thus, exposure to ionizing radiation would likely result in continued cyclicity until the pool of larger sized follicles was exhausted (Mattison and Shulman, 1980). Since the smaller follicles would already be depleted, premature ovarian failure would eventually occur. Additionally, since ionizing radiation targets rapidly dividing cells, \textit{in utero} exposure could affect the number of oocytes formed in female offspring and compromise reproduction (Mattison and Schulman, 1980).

Numerous drugs affect reproductive function. The anticancer agent, busulfan, was the first alkylating agent shown to cause altered ovarian function in rodents and humans (Mattison and Shulman, 1980). In some cases, there was a temporary loss of fertility, while in others, the result was permanent infertility. Several studies reported that premenopausal women being treated with busulfan for chronic myelogenous leukemia became amenorrheic within 3-6 months of receiving chemotherapy at doses of 0.5 to 14 mg/d (Schilsky et al., 1980). Thus, it was concluded that growing and pre-ovulatory follicles were more sensitive to the effects of busulfan, compared to the smaller follicles. Another alkylating agent, cyclophosphamide, is widely used in the treatment of various types of cancers. This drug, which must be activated to a chemically reactive metabolite, also targets growing and pre-ovulatory follicles (Plowchalk and Mattison, 1991). Thus, depending on the number of follicles present in the ovary at the onset of treatment and the treatment duration, temporary or permanent infertility may result. For example, Mattison and Schulman (1980) found that “in patients with breast cancer, the dose necessary to produce permanent amenorrhea appears to be proportional to the total
number of oocytes: younger women require larger doses than do older women."

Permanent cessation of menses occurred after a mean total dose of 5.2 g cyclophosphamide in all patients 40 years of age and older (Schilsky et al., 1980).

Treatment with cyclophosphamide in prepubertal females did not result in ovarian damage or a delay in menarche (the onset of menstruation). Combination chemotherapy will likely result in permanent ovarian dysfunction, however the effects are not currently clear, due to inadequate clinical documentation (Chapman, 1983). Amenorrhea has also been noted with the use of the atypical antipsychotic drug, risperidone. Ernst and Goldberg (2002) suggest that the menstrual dysfunction seen following treatment is due to elevated prolactin levels, which in turn, inhibits LH and ovulation.

Environmental agents including heavy metals and pollutants such as organochlorine pesticides, constituents in cigarette smoke (polycyclic aromatic hydrocarbons), and occupational/industrial chemicals also have reproductive specific effects. For example, exposure to lead has been associated with a variety of chromatid and chromosome breaks. Toxicity has been characterized by follicular atresia in rodents and non-human primates (Mattison et al., 1990). Additionally, lead poisoning in humans has been associated with reduced fertility, miscarriages, and stillbirths (Gilfillan, 1965). The organochlorine pesticides, such as methoxychlor and chlordecone, alter the reproductive system in rats. Methoxychlor exposure accelerates vaginal opening, causes irregular estrous cyclicity, blocks implantation, reduces fertility, and decreases litter size. Chlordecone causes persistent estrus and anovulation in rats (Sharara et al., 1998). "At least 60 toxic compounds are among the 4000 chemical constituents identified in tobacco
smoke, and include the polycyclic aromatic hydrocarbons” (Shiverick and Salafia. 1999). Epidemiological studies suggest that female smokers reach menopause 1 to 1.5 years earlier than non-smokers (Shiverick and Salafia. 1999). The prototype PAHs, 7,12-dimethylbenzanthracene (DMBA) and benzo(a)pyrene (B(a)P) destroy oocytes in mice and alter the formation of the corpus luteum (Mattison. 1980a). More recent in vitro studies suggested that DMBA produced a dose-dependent increase in apoptosis in mouse and human granulosa cells (Tilly et al., 1997). Furthermore, in humans, B(a)P-DNA adducts have been detected in granulosa cells of women smokers who were undergoing in vitro fertilization. The reproductive effects of the industrial compound, 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), have also been characterized. Studies in rats suggested multiple mechanisms of action. For instance, TCDD may affect the hypothalamic-pituitary-ovarian axis, thus, causing estrous cycle and ovulation alterations, or TCDD may act directly on the ovary, inhibiting 17β-estradiol production in the granulosa cells (Li et al., 1995; Moran et al., 2000). Additionally, decreases in fertility, abnormal vaginal development, decreased ovarian weight, and induction of cystic endometrial hyperplasia were reported in rats following in utero exposure to TCDD. In monkeys, TCDD “alters hormone levels, decreases fertility, and causes endometriosis” (Sharara et al., 1998). The occupational chemical, 2-bromopropane (2-BP), also causes destruction of ovarian follicles in rats. Chronic exposure (9 weeks) to 2-BP (300 and 1000 ppm) caused a significant reduction in the number of primordial, growing, and antral follicles in a dose-dependent manner. Follicle loss is likely via apoptosis (Yu et al., 1999). Another occupational chemical, 1,3-butadiene (BD), is used in the synthetic
rubber industry and has also been reported in cigarette smoke and automobile exhaust. Female mice, exposed to BD for 2 years in inhalation studies, developed ovarian atrophy, granulosa cell hyperplasia, as well as granulosa cell tumors (benign and malignant; Melnick et al., 1990). Shorter dosing regimens (30 days) with the monoepoxide (BMO) and diepoxide (BDE) metabolites of BD resulted in the loss of small and growing pre-antral follicles in mice (Doerr et al., 1995). Additional studies by Doerr et al (1996) showed that BMO was not ovotoxic in the rat, whereas BDE was ovotoxic. Thus, it was concluded that BDE is the ultimate ovotoxic metabolite and that differences in the metabolism of BMO and BDE may at least partially explain the species difference in susceptibility. Similarly, another example of a toxicant that targets pre-antral (primordial and small primary) follicles is the occupational chemical, 4-vinylcyclohexene, or VCH. This chemical is of particular interest in our laboratory.

**Ovarian Metabolism:**

Although the liver is the primary organ involved in metabolic reactions of drugs, as well as xenobiotics, extrahepatic organs can also contribute to the bioactivation and/or detoxification reactions of such compounds (Sipes and Gandolfi, 1991). Various methodologies, including immunoblotting techniques with antibodies and *in vitro* incubations with the tissue/enzyme and chemical of interest, have resulted in an explosion of information regarding extrahepatic metabolism. Numerous enzymes including the CYP450 isoforms, epoxide hydrolases, glutathione S-transferases, UDP-glucuronosyl-transferases, sulfotransferases, N-acetyltransferases, and methyltransferases
have been identified in a multitude of tissues. The human tissue distribution of these enzymes is broad, including the gastrointestinal tract mucosa, kidney, lung, brain, skin, testis, and ovary, to name a few (Krishna and Klotz, 1994).

Specifically in the ovary, Mukhtar et al (1978) evaluated the postnatal development of microsomal CYP 450, microsomal epoxide hydrolase (mEH), and glutathione S-transferase in ovarian microsomes and cytosol obtained from rats. Total CYP 450 content, determined by binding of carbon monoxide (Omura and Sato, 1964), was approximately 0.02 nmol/mg of protein at 12 days of age and gradually increased to a maximum of approximately 0.07 nmol/mg of protein at 60 days of age. Utilizing benzo(a)pyrene-4,5-oxide as a substrate, ovarian mEH activity was determined in which activity was approximately 0.3 nmol/min/mg of protein at 12 days of age. By day 40, activity had increased to about 0.85 nmol/min/mg of protein, however, activity decreased to levels of about 0.7 nmol/min/mg of protein by day 60, after which activity levels remained fairly constant. Styrene-7,8-oxide and benzo(a)pyrene-4,5-oxide were used as substrates to determine cytosolic glutathione S-transferase activity. At day 12, enzyme activity was similar for both substrates (approximately 20 nmol/min/mg or protein). Additionally, the patterns of glutathione S-transferase activity were similar for both substrates, in which activity peaked at 35 days of age, then gradually decreased and remained constant by 140 days of age. However, specific activity utilizing styrene oxide as a substrate was greater compared to benzo(a)pyrene oxide at every time point (105 nmol/min/mg of protein versus 50 nmol/min/mg of protein, respectively, 35 days; 75 nmol/min/mg of protein versus 45 nmol/min/mg of protein, respectively, 180 days).
These studies verified that several enzymes important in many metabolic reactions are present and are functional in the rat ovary, and thus, may contribute to ovarian metabolism of a variety of agents.

Ovarian metabolism is thought to play a critical role in the resulting ovotoxic and carcinogenic effects of several polycyclic aromatic hydrocarbons, including benzo(a)pyrene (B(a)P), 7,12-dimethylbenzanthracene (DMBA), and 3-methylcholanthrene (3-MC). Since bioactivation of these compounds is required to form the toxic metabolites, induction of the ovarian specific responses was presumed to be dependent on metabolism (Figure 1.2). Studies by Jull et al. (1968) supported the role of ovarian metabolism in the toxicity of DMBA because when whole ovaries were treated with DMBA in vitro and subsequently transplanted into castrated mice that were not previously exposed to DMBA, ovarian granulosa cell tumors formed. Later studies also revealed formation of ovarian granulosa cell tumors in mice following exposure to B(a)P and 3-MC (Jull et al., 1973).

Mattison and Thorgeirsson (1979) further evaluated the in vivo effects following dosing (i.p.) with B(a)P, DMBA, and 3-MC on primordial follicle toxicity in mice. Their studies revealed a correlation between induction in ovarian aryl hydrocarbon hydroxylase activity and a decrease in the number of primordial follicles. DMBA was the most potent chemical in terms of follicle destruction, followed by 3-MC and B(a)P. These effects were dose and time dependent. Additionally, follicle loss was inhibited when α-naphthoflavone (ANF), a known CYP 450 inhibitor (Weibel et al., 1971), was co-administered with the respective PAH. However, these studies did not elucidate the
Figure 1.2: Prototypical metabolic scheme for the polycyclic aromatic hydrocarbon, DMBA. The parent compound, DMBA, is bioactivated via cytochromes P450 and mEH to form the ultimate toxic metabolite, DMBA-3,4-diol-1,2-epoxide. Scheme adapted from Miyata et al., 1999.
direct role of ovarian metabolism in the resulting ovotoxicity. Thus, Shiromizu et al (1984) investigated the direct effects of intraovarian injection of B(a)P on primordial follicle number and ovarian aryl hydrocarbon hydroxylase activity. Following unilateral injection of B(a)P into one of two ovaries in mice, follicle destruction was only evident in the B(a)P-treated ovary. This primordial follicle destruction was dose and time dependent. Follicle loss was also inhibited when mice were pre-treated with ANF, via the i.p. route of administration. Additionally, aryl hydrocarbon hydroxylase activity was induced in those ovaries injected with B(a)P, relative to the other ovary that was injected with corn oil, serving as the vehicle control. These studies provided direct evidence for the role of ovarian metabolism in B(a)P-induced ovotoxicity.

Interestingly, the ovotoxic effects following PAH exposure are species specific. Following PAH exposure in mice, primordial follicles are destroyed and granulosa cell tumors develop after complete oocyte destruction (Jull. 1973). However, rats are more resistant to both follicle loss and tumor formation, compared to the mouse. This difference in species specificity is thought to be due to differences in metabolism (hepatic and/or ovarian), since the PAHs are not directly toxic and thus require metabolic activation to the bioactive metabolites. Mattison (1979b) suggested that the "decreased oocyte toxicity in the rat may reflect differences in monooxygenases, epoxide hydrases, or transferases." Studies utilizing B(a)P as a substrate suggested that rat and mouse ovarian monooxygenases differ in the relative amounts of products formed. For instance, ovarian AHH activity in naïve mice and rats was 7.0 pmol/min/mg of protein and 3.1 pmol/min/mg of protein, respectively. Following in vivo treatment with 3-MC, activity
was induced in both species, however, activity was still greater in the mouse compared to the rat (19.6 pmol/min/mg of protein compared to 10.6 pmol/min/mg of protein, respectively). Furthermore, when B(a)P metabolism is directly evaluated, via metabolite formation and identification by HPLC, total B(a)P metabolism is greater in the mouse (74.94±12.1 fmol/min/mg of protein) compared to the rat (23.39±6.6 fmol/min/mg of protein). Again, pretreatment with 3-MC induced B(a)P metabolism in both species, however, the mouse still exhibited a greater ability to metabolize B(a)P compared to the rat (296.86±44.1 fmol/min/mg of protein versus 123.97±14.8 fmol/min/mg of protein, respectively). Since the suspected ovotoxic and carcinogenic metabolite of B(a)P is the diol-epoxide metabolite (7,8-diol-9,10-oxide), which requires both epoxide hydrolase and monooxygenase activity, the differences in species specificity is not clear cut. Rather, differences in toxic and nontoxic pathways of PAH metabolism are likely. Additionally, since multiple isoforms exist for both the monooxygenases, as well as the epoxide hydrases, it is not surprising that there are inherent species differences, thus generating different B(a)P metabolic profiles in the mouse and rat ovary. “The lower rate of production of the 7,8-diol by rat ovarian monooxygenase is consistent with the greater resistance of the rat ovary to PAH ovotoxicity and ovarian carcinogenicity” (Mattison et al., 1979c). Although differences in metabolic enzymes in the ovary may partially explain differences in the species specificity to the PAHs, Mattison also suggests that other factors may be involved. For instance, mice are about ten times more susceptible to primordial oocyte destruction by ionizing radiation, compared to the rat. This difference in susceptibility is thought to be due to differences in oocyte chromatin, in which it is
more diffusely organized in the mouse compared to the rat, thus making mouse oocytes more sensitive to destruction by ionizing radiation (Mattison, 1979b). Genetic differences in different strains of the same species may also affect the outcome following PAH exposure. Studies by Mattison and Nightingale (1980) showed that C57BL.6N (B6N) mice were sensitive to the ovotoxic effects of PAHs, whereas a different mouse strain, DBA/2N (D2N) was resistant. They concluded that the basic differences in sensitivity to oocyte destruction by the PAHs were due to differences in the balance of bioactivation and detoxification, as well as differences in repairing damaged DNA in oocytes.

Studies by Bengtsson et al (1983), further characterized the relative contribution of ovarian metabolism of B(a)P in the rat ovary, compared to metabolism by the liver and adrenal gland. Metabolite patterns were determined by HPLC, which were similar in the ovary and adrenal gland. Both organs produced similar amounts of 9,10-diol and 7,8-diol, while the adrenal gland produced more of the 3-hydroxy and 9-hydroxy metabolites. The liver profile was very different, in which quinone metabolites were abundantly produced. These metabolites were not seen in the ovary or adrenal gland. The high affinity of the enzymes in the ovary and adrenal gland suggests that the ovary may play a substantial role in bioactivation of xenobiotics. The ovary may be more sensitive to the effects of these chemicals, compared to the liver, thus making the contribution of ovarian metabolism even more important.

To further elucidate the regulatory mechanism of DMBA hydroxylase in the rat ovary, Bengtsson et al (1987) evaluated endogenous factors. Estrogen (estradiol, E2)
increased aryl hydrocarbon hydroxylase activity in the granulosa and theca cells, likely through induction. The synthetic estrogen, diethylstilbestrol (DES) also increased aryl hydrocarbon hydroxylase activity. Additionally, the gonadotropins FSH and LH stimulated this enzyme activity via increased proliferation of the granulosa and theca cells. Progesterone, prolactin, and growth hormone had no effect. In the presence of the E₂ antagonist, tamoxifen (which inhibits cell proliferation), ovarian aryl hydrocarbon hydroxylase activity was only partially blocked, suggesting that endogenous regulation is only part of the answer. Bengtsson et al (1987, 1992) also concluded that most of the enzyme activity was likely in the granulosa and theca cells of large, antral follicles. In humans, DMBA metabolism also appears to be greatest in the granulosa cells. However, there is no change in the rate of DMBA metabolism in the presence of FSH or LH (Bengtsson et al., 1988). Thus, the regulation appears to be different for the rat and the human.

As multiple isoforms for the Cyp 450 enzymes were discovered, the identity of the isoforms in the ovary became of particular interest, due to their involvement in PAH metabolism, toxicity, and carcinogenicity. Studies by Bengtsson et al (1990) evaluated the identity of several Cyp 450 enzymes in the rat ovary. In ovarian microsomes obtained from rats, it was concluded that none of the isozymes evaluated (CYP 1A1, CYP 2B1/2, CYP 2A1/2, and CYP 3A) were present in sufficient amounts to play a significant role in PAH metabolism, as evaluated by Western blotting. Although the involvement in PAH metabolism is not known, several CYP 2C isoforms have been detected in rat ovarian microsomes. Interestingly, this subfamily of enzymes is thought
to be involved in metabolism of xenobiotics, as well as endogenous steroids and arachidonic acid. Studies by Zaphiropoulos et al (1995) further evaluated these isoforms at the mRNA level in porcine ovaries. Based on structural identity, two forms were identified. Unfortunately, it is not known how this information correlates to levels of the corresponding proteins, or whether these isoforms participate in xenobiotic metabolism.

4-Vinylcyclohexene – Use, Production, and Exposure:

4-Vinylcyclohexene (VCH) is a dimer of the occupational chemical, 1,3-butadiene (BD). It can form spontaneously in the manufacture of BD (Keller et al., 1997). VCH is also used as an intermediate in the manufacture of flame retardants and plasticizers, as well as a solvent in the manufacture of 4-vinylcyclohexene diepoxide (VCD).

Individuals are likely to be exposed to VCH in the occupational and/or industrial setting. Workers could potentially be exposed to VCH during the production of 1,3-butadiene-based rubber, rubber vulcanization in the manufacture of shoe soles, tires, and other rubber products, as well as in the manufacture of flame retardants and insecticides. However, production policies in these venues have mandated that these chemical processes are performed in closed vessels. Thus, human exposure to VCH is limited, with exception of accidental spills and leaks (IARC, 1994). Nonetheless, air concentrations of VCH have been measured in the workplace. For instance, levels of 0.03 to 0.21 mg/m³ were measured in an Italian shoe plant, levels of 0 to 0.003 mg/m³ were measured in an Italian tire factory, and levels of 0.24 to 0.43 mg/m³ were measured in an American tire curing room (IARC, 1994; Rappaport and Fraser, 1977). After these
short term air concentration studies, the American Conference of Governmental
Industrial Hygienists proposed and later established a threshold value for VCH exposure
in an 8 hour period in 1992. The limit value is 0.4 mg/m³. This same group also
classified VCH as a suspected human carcinogen (IARC. 1994). Currently, there are no
epidemiological studies to relate human exposures to toxicity and/or cancer.

VCH Structure and Pharmacokinetics:

VCH is a colorless liquid. It has a formula weight of 140.2 g/mol. The structure
contains two double bonds, one at the 1,2 position on the cyclohexene ring, and the other
at the 7,8 position on the vinyl side chain (Figure 1.3).

ABSORPTION

In rodent studies, VCH has been dosed via oral (p.o.) gavage (Collins and Manus, 1987; Collins et al., 1987), intraperitoneal (i.p.) injection (Smith et al., 1990b), dermal
application (Chhabra et al., 1990; NTP. 1989), and inhalation (Bevan et al., 1996). As
evidenced by the pharmacokinetic profiles, absorption was adequate following all routes
of administration.

DISTRIBUTION

Studies by Smith et al (1990a) evaluated the disposition of a single dose (400
mg/kg; p.o. gavage) of [¹⁴C]-VCH in female B6C3F₁ mice. [¹⁴C]-VCH was widely
distributed, however, by 24 h, no tissue contained more than 1% of the total dose. The
tissue distribution of $[^{14}\text{C}]-\text{VCH}$ was evaluated 1, 2, 4, and 6 h after the dose. The highest concentrations for $[^{14}\text{C}]-\text{VCH}$ for all examined tissues was at 2 h. Adipose tissue contained the highest concentration of $[^{14}\text{C}]-\text{VCH}$ (~ 4 nmol VCH/mg tissue), compared to all other tissues (liver, ~ 0.65 nmol VCH/mg; skin, ~ 0.45 nmol VCH/mg; muscle, ~ 0.15 nmol VCH/mg; blood, ~ 0.07 nmol VCH/mg; ovary, ~ 0.4 nmol VCH/mg).

Interestingly, distribution of radioactivity in the ovary was negligible when expressed as a percent of the total dose.

**METABOLISM**

The two double bonds in the structure of VCH make it a prime candidate for oxidative metabolism by the cytochrome P450 enzymes. Due to the high concentration of these enzymes in the liver, VCH metabolism is primarily thought to be hepatic. In the hepatic metabolic scheme proposed by Keller et al. (1997; Figure 1.3), VCH is first bioactivated by the cytochrome P450 (Cyp 450) enzymes to form one of two possible monoepoxide metabolites, either at the 1,2 position (1,2-VCM) or the 7,8 position (7,8-VCM).

Following a single dose of VCH (800 mg/kg; i.p.) in mice, studies by Smith et al. (1990a) showed that the blood concentration of 1,2-VCM was highest at 2 h (41 nmol/ml).

Additionally, the rate of formation of 1,2-VCM was 9.1 nmol/min/mg of protein, as evaluated in hepatic microsomes. Levels of 7,8-VCM could not be detected after a single dose. Following the first epoxidation reaction, the monoepoxides can, in theory, undergo one of three fates. The remaining double bond can undergo subsequent bioactivation via Cyp 450 to form the diepoxide metabolite, VCD, the monoepoxides can be detoxified.
Figure 1.3: Proposed scheme for hepatic metabolism of VCH. The parent compound, VCH, is bioactivated via cytochromes P450 to form the mono and di-epoxide metabolites. Detoxification via hydrolysis of epoxides is via microsomal epoxide hydrolase. (Keller et al., 1997)
Figure 1.3

[Diagram of chemical reactions and structures, including molecules labeled as TETROL, ych, 1.2-VCM, 7,8-VCM, mEH, P450, and VCD]
presumably by microsomal epoxide hydrolase (mEH) to form the diol metabolites (VCH 1.2-diol or VCH 7,8-diol). or the monoepoxides can be detoxified by forming conjugates with glutathione. Studies by Doerr et al (1995) detected VCD in the circulation of mice following a single dose of VCH. VCD can further be detoxified via mEH to form the diol-epoxides (7,8-epoxide 1,2-diol or 1,2-epoxide 7,8-diol) and the ultimate tetrol metabolite (4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane). or VCD can be detoxified by forming glutathione conjugates. As an alternate metabolic pathway, the diol metabolites can also be metabolized via Cyp 450 to the diol-epoxide, before subsequent detoxification to the tetrol or glutathione conjugate.

A number of studies have focused on which Cyp 450 isoforms bioactivate VCH. Initial in vitro studies in hepatic microsomes by Smith et al (1990c), suggested that the Cyp 450 isoforms involved in VCH metabolism were Cyp 2A and Cyp 2B. VCH epoxidation was partially inhibited in microsomes obtained from chloramphenicol-treated mice and was induced in microsomes obtained from phenobarbital-treated mice. Studies by Doerr et al (1999), showed hepatic induction of Cyp 2A, Cyp 2B, and Cyp 2E1 by Western blot analysis, following repeated in vivo dosing of mice with VCH.

Studies by Fontaine et al (2001a, 2001b) further elucidated the roles of specific Cyp 450 enzymes involved in VCH and 1,2-VCM bioactivation. As evaluated by Western blot analysis in mice, Cyp 2A and Cyp 2B were induced following in vivo dosing with VCH, whereas only Cyp 2B was induced following in vivo dosing with 1,2-VCM. Specific activities for Cyp 2A and Cyp 2B were induced after in vivo dosing with
either VCH or 1.2-VCM. In these studies, Cyp 2E1 was not induced after *in vivo* dosing with either VCH or 1.2-VCM.

Because Cyp 2E1 is known to metabolize low molecular weight compounds (Guengerich *et al*., 1991; Koop, 1992; Wang *et al*., 1995), including the VCH-related olefin, BD (Jackson *et al*., 1999), Fontaine *et al* (2001b) evaluated the role of Cyp 2E1 in VCH metabolism. In purified Cyp 2E1 microsomes from humans, also known as “supersomes,” VCH and 1.2-VCM were metabolized, however enzyme levels of Cyp 2E1 were not increased. To study the role of Cyp 2E1 in mice, microsomes obtained from Cyp 2E1 deficient mice were incubated with VCH and metabolites measured and identified. Interestingly, the same amount of 1.2-VCM and 7.8-VCM were formed from VCH in hepatic microsomal incubations from Cyp 2E1 deficient and wild type mice, suggesting that Cyp 2E1 is not involved in VCH epoxidation in the mouse liver.

**EXCRETION**

Kinetic studies by Smith *et al* (1990a) revealed that 24 h after a single dose of $[^14]C$-VCH (p.o. gavage), approximately 97% of the total dose was recovered. Approximately 58% of the dose was excreted in the urine, while 3% of the dose was excreted in the feces. About 31% of the dose was expired into the air, 2% of the dose was in the tissues, and 3% of the dose was recovered from the cage wash.

When a single dose of $[^14]C$-VCD (100 mg/kg; i.p.) was administered to mice, approximately 80% of the dose was excreted in the urine within 24 h (30% of the dose within 6 h). Fecal elimination was a minor route of excretion (4-5%; Salyers, 1995).
However, the tetrol metabolite was only found to be approximately 5-10% of the urinary metabolite profile. Thus, Salyers also proposed that some of the metabolites were glutathione conjugates. In rats, approximately 90% of the dose was excreted in the urine within 24 h (70% of the dose within 6 h). 4-5% of the dose was excreted in the feces, and the tetrol was the major urinary metabolite, which accounted for approximately 60% of the dose.

Role of Biotransformation in VCH-Induced Ovotoxicity:

VCH is a reproductive toxicant in mice. Bioactivation of VCH by the Cyp 450 enzymes to the diepoxide metabolite (VCD) is required to produce the ovarian-specific toxic effects, which involves targeting and destruction of immature, small pre-antral follicles (Doerr et al., 1995; Smith et al., 1990b). Although the exact mechanism of VCH induced-ovotoxicity is not known, metabolism (bioactivation and detoxification) is a key component, and is thought to be at least partially responsible for a species specific response, in which VCH is ovotoxic in the mouse, but not the rat. The VCH-monoepoxides and VCD are ovotoxic in both species (Smith et al., 1990a; Smith et al., 1990b). Specifically, it is thought that the mouse may be more sensitive to the effects of VCH compared to the rat because of a greater ability to bioactivate VCH (via CYP 450) and a lesser ability to detoxify VCD (via mEH) to the non-toxic tetrol metabolite (4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane). This is further supported by Fontaine et al. (2001a) and Salyers et al. (1993). Fontaine et al. (2001a) showed that hepatic protein levels and specific activities for Cyp 2A and Cyp 2B were induced in mice, but not rats.
following *in vivo* dosing with VCH or 1.2-VCM. Studies by Salyers *et al* (1993) showed that 6 h after receiving a single dose of [\(^{14}\)C]-VCD, rats excreted 70% of the dose in the urine, whereas mice only excreted 30% of the dose in the urine.

Studies by Doerr *et al* (1995) tested whether several related olefins to VCH that could be metabolized to either a monoepoxide or diepoxide metabolite could cause follicle loss in mice. Only those compounds that could form a diepoxide metabolite caused destruction of follicles. Thus, these structure activity studies verified that VCD is the ultimate ovotoxic metabolite responsible for destruction of immature, small pre-antral follicles.
CHAPTER 2

GENERAL OVERVIEW AND METHODS

STATEMENT OF PROBLEM:

The role of ovarian metabolism in PAH-induced ovotoxicity and/or carcinogenicity forces one to consider the role of ovarian metabolism for other ovotoxic compounds, including 4-vinylcyclohexene, or VCH. Since bioactivation of VCH to the diepoxide metabolite is crucial for the resulting ovarian toxicity, it is important to understand whether or not the ovary can participate in such reactions. It is of equal importance to understand the role of the ovary in detoxification reactions.

Although Bengtsson and colleagues have suggested that ovarian metabolism of the PAHs occurs in the granulosa cells of rat ovaries, the specific follicle sizes involved are not known. This is because studies were performed in microsomes from whole ovaries. Furthermore, the distribution and identity of the Cyp 450 isoforms in the mouse ovary is not known.

Evaluating the ovarian gene and protein expression of various metabolic enzymes thought to be involved in the hepatic metabolism of VCH/VCD will help us understand the role of ovarian metabolism in VCH-induced ovotoxicity in B6C3F1 mice.
RESEARCH OBJECTIVES:

The working hypothesis of this dissertation is that ovarian metabolism plays a role in VCH-induced ovotoxicity in B6C3F1 mice, whether it be through an increased ability to bioactivate VCH (via Cyp 450) and/or a decreased ability to detoxify VCD (via the detoxification enzyme, mEH). The major research objectives to test this hypothesis are described below.

1) The first objective was to determine the presence and distribution of mRNA and total protein for various Cyp 450 isoforms (thought to be involved in the hepatic metabolism of VCH) in the mouse ovary. Basal expression of mRNA and total protein was evaluated by real-time, reverse transcriptase polymerase chain reaction and confocal microscopy, respectively. To determine whether or not the enzymes were functional, catalytic assays were performed, using model substrates for the specific Cyp 450 isoforms of interest. The effects of *in vivo* dosing with either VCH or VCD on this gene expression, protein distribution, and functional activity were also determined.

2) The second objective was to determine the presence and ovarian distribution of mRNA and total protein for the detoxifying enzyme, mEH, in the mouse ovary. Basal expression of message and total protein was evaluated by real-time, reverse transcriptase polymerase chain reaction and confocal microscopy, respectively. To determine whether or not the enzyme was functional, catalytic assays were performed, using the model substrate, *cis*-stilbene oxide. The effects of *in vivo* dosing (1 single dose versus 15 daily, consecutive doses) with either VCH or VCD on this gene expression, protein distribution, and functional activity were also determined.
3) The third objective was to determine the role of the ovary in bioactivation and detoxification reactions of 1,2-vinylecyclohexene monoepoxide (VCM) or VCD, respectively. To eliminate the contribution of hepatic metabolism, an in vitro culture system utilizing whole ovaries was used to evaluate follicle loss as a physiological endpoint. Whole ovaries from 4 day old mice were incubated in vitro for 15 days. Following incubation, the ovaries were fixed for histology and the number of follicles (primordial, small primary, large primary) were counted.

4) The fourth objective was to evaluate the specific activities of the Cyp 450 enzymes in rat ovaries, to further elucidate the differences in species susceptibility to VCH, VCM, and VCD. Following 15 days of repeated dosing, specific activities for various Cyp 450 isoforms were determined in whole ovaries using model substrates.

MATERIALS AND METHODS:

Reagents: Medium 199 (M199), Hank’s buffered saline (without CaCl₂, MgCl₂, MgSO₄, or phenol red), Ham’s F-12/Dulbecco’s modified eagle’s medium (1:1), albumax, penicillin, streptomycin, and custom designed primers were purchased from In Vitrogen (Grand Island, NY). VCH (racemic mixture; purity 95-99%), dichloromethane, and 7-hydroxy coumarin were purchased from Aldrich Chemical Co. (Milwaukee, WI). 1,2-VCM (mixture of isomers, composition unknown; purity > 98%), 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-hydroxyethyl)piperazine-N’-(2-ethanesulfonic acid) (HEPES).
NaCl, ethylenediaminetetraacetic acid (EDTA), Tris, triton X, glycerol, sodium dodecyl sulfate (SDS), sodium fluoride, phenylmethanesulfonyl fluoride (PMSF), leupeptin, aprotinin, potassium phosphate, potassium chloride, magnesium chloride, sodium pyrophosphate, butylated hydroxytoluene (BHT), NADPH, coumarin, p-nitrophenol, p-nitro catechol, hydrochloric acid, trichloroacetic acid, sodium borate, ribonuclease A, ascorbic acid, and transferrin 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).

Deoxynucleotidetriphosphate (dNTP) mix, MgCl₂, and enzyme diluent were purchased from Idaho Technologies (Salt Lake City, UT). Advantaq 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 bicinchoninic acid (BCA) protein assay kit was from Pierce (Rockford, IL). Cyp 2A6 antibody (rabbit anti-human) was purchased from Affinity BioReagents, Inc. (Golden, CO). Cyp 2B1 antibody (goat anti-rat) and Cyp 2E1 antibody (goat anti-rat) were purchased from Gentest (Woburn, MA). CytoScint and non-radioactive cis-stilbene oxide were from ICN Radiochemicals (Costa Mesa, CA). The [³H] cis-stilbene oxide was a generous gift from Dr. Bruce Hammock (UC Davis, CA). The mEH antibody (goat anti-rabbit) was purchased from Detroit R and D (Detroit, MI). Rabbit anti-goat secondary antibody (Cyp 2E1, Cyp 2B1), goat anti-rabbit secondary antibody (Cyp 2A6), horse anti-goat
secondary antibody (mEH), and Cy-5-streptavidin were purchased from Vector (Burlingame, CA). Millicell-CM filter inserts were from Millipore (Bedford, MA).

**Animals:** Day 21 (d 21) female B6C3F1 mice, Fischer-344 rats, or late gestation C57BL/6J mice (crossed with C3H males to give B6C3F1 pups), were purchased from Harlan Laboratories (Indianapolis, IN), and maintained in the University of Arizona Animal Care Facility for 1 week prior to study. Mice (d 21) and rats (d 21) were housed in plastic cages, 5 animals per cage, whereas 1 pregnant mouse was housed per cage. All animals were maintained on 12 h light/12 h dark cycles in a controlled temperature of 22±2°C. The animals were provided a standard diet with *ab libitum* access to food and water. Pregnant mice were monitored for litters daily, and pups were utilized for *in vitro* culture experiments when they were 4 days of age. All animal experiments were approved by the University of Arizona’s Institutional Animal Care and Use Committee.

**In Vivo Animal Dosing:** Day 28 (d 28) female mice or rats (8-10 animals/treatment group) were weighed and administered (intraperitoneal: ip) 1 single dose or 15 daily, consecutive doses (2.5 μl/g of body weight) of either sesame oil (vehicle) or sesame oil containing VCH (7.4 mmol/kg/d), 1,2-VCM (2.74 mmol/kg/d), or VCD (0.57 mmol/kg/d). The species, strains, equitoxic doses, routes of administration, and dosing time courses were based on previous studies (NTP, 1989; Smith *et al.*, 1990b; Borman *et al.*, 1999). Animals were killed by CO2 inhalation 4 h following the final dose.
Follicle Isolation: Mouse ovaries were removed and the oviduct and excess fat were trimmed away. Ovaries were minced and gently dissociated (40°C, 20 minutes) 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 resulting suspension was filtered through a 250 μm 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) pre-antral follicles and interstitial cells (Int) were further hand sorted using calibrated Pasteur pipettes into distinct populations (Flaws et al., 1994a; Figure 2.1). Tissue from the distinct ovarian fractions was stored (-80°C) until further use. Because F1 and F2 follicles are hand sorted during the procedure, these follicular fractions 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). In both instances, 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 obtained from mice utilizing the RNAqueous® 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).
Figure 2.1: Schematic of Ovarian Follicle Isolation. Whole ovaries were minced and gently dissociated (40°C, 20 minutes) in M199 containing collagenase, DNase, and BSA. The resulting suspension was filtered through a 250 μm screen via vacuum suction to exclude antral follicles (> 250 μm; F3). The filtered follicles were further hand sorted into F1 follicles (25-100 μm), F2 follicles (100-250 μm), and Int cells.
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).

Realtime 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 realtime PCR. The LightCycler® quantifies the amount of PCR product generated by measuring the dye (SYBR green), which fluoresces when bound to double stranded DNA. A standard curve was generated from 1:5 serial dilutions of purified PCR product (Cyp 2E1, Cyp 2A, Cyp 2B, mEH, or 18S rRNA). Custom designed primers were utilized (Table 2.1).
<table>
<thead>
<tr>
<th>Metabolic Enzyme</th>
<th>Forward Primer 5'→3'</th>
<th>Reverse Primer 5'→3'</th>
<th>Product Size (Base Pairs)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 2E1</td>
<td>GTC TTT AAC CAA GTT GGC AA</td>
<td>CCA ATC AGA AAG GTA GGG TC</td>
<td>340</td>
<td>Freeman et al., 1992</td>
</tr>
<tr>
<td>Cyp 2A</td>
<td>ATT GAC CCC ACC TTC TAC CT</td>
<td>CAG TAT TGG GGT TCT TCT TCT CC</td>
<td>352</td>
<td>Squires and Negeshi, 1988</td>
</tr>
<tr>
<td>Cyp 2B</td>
<td>CTC TTC CAG TGC ATC AC</td>
<td>GGA ACT CCT CGA CTA CAT TG</td>
<td>229</td>
<td>Marc et al., 1999</td>
</tr>
<tr>
<td>mEH</td>
<td>GGG TCA AAG CCA TCA GCC A</td>
<td>CCT CCA GAA GGA CAC CAC TTT</td>
<td>156</td>
<td>NCBI, 1997</td>
</tr>
</tbody>
</table>

Table 2.1: Custom Designed Primer Sequences for Metabolic Enzymes.

Amplification conditions for metabolic enzymes of interest, in addition to 18 S rRNA, are presented in Table 2.2.

<table>
<thead>
<tr>
<th>Metabolic Enzyme</th>
<th>Denaturing Conditions</th>
<th>Annealing Conditions</th>
<th>Extension Conditions</th>
<th>Cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 2E1</td>
<td>95°C/0 sec</td>
<td>57°C/0 sec</td>
<td>72°C/10 sec</td>
<td>50</td>
</tr>
<tr>
<td>Cyp 2A</td>
<td>95°C/0 sec</td>
<td>68°C/0 sec</td>
<td>72°C/12 sec</td>
<td>50</td>
</tr>
<tr>
<td>Cyp 2B</td>
<td>95°C/0 sec</td>
<td>64°C/0 sec</td>
<td>72°C/16 sec</td>
<td>50</td>
</tr>
<tr>
<td>mEH</td>
<td>95°C/0 sec</td>
<td>65°C/0 sec</td>
<td>72°C/6 sec</td>
<td>50</td>
</tr>
<tr>
<td>18 S</td>
<td>95°C/0 sec</td>
<td>57°C/0 sec</td>
<td>72°C/16 sec</td>
<td>40</td>
</tr>
</tbody>
</table>

Table 2.2: RT-PCR Amplification Conditions for Different Metabolic Enzymes of Interest and 18 S rRNA.
A standard curve was generated from 1:5 serial dilutions of purified PCR product. An aliquot of each dilution was used as PCR template to generate a standard curve. Arbitrary numbers were assigned to each standard and experimental samples were extrapolated from the standard curve. 18S rRNA was measured in each sample as an internal standard. Final values were expressed as a ratio of mRNA expression:18 S rRNA.

Confocal Microscopy: Ovaries from 4 day old mice (untreated) or from d 42 mice (treated \textit{in vivo} for 15 d; 2 animals/group), were removed following euthanization, and oviduct and excess fat trimmed away. Ovaries were fixed for 4 h in 10% buffered formalin, dehydrated, and embedded in paraffin. Every 7th section (5 μm thick) throughout the ovary was prepared and deparaffinized (approximately 24 sections/group). Sections were incubated with primary antibodies and secondary biotinylated antibodies, according to Table 2.3 and Table 2.4, respectively, followed by Cy-5-streptavidin (1 h: 1:50 dilution; 37°C; 60 min).

<table>
<thead>
<tr>
<th>Metabolic Enzyme</th>
<th>Origin of Antibody</th>
<th>Dilution of Antibody</th>
<th>Incubation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 2E1</td>
<td>goat anti-rat</td>
<td>1:50</td>
<td>37°C, 60 min</td>
</tr>
<tr>
<td>Cyp 2A</td>
<td>rabbit anti-human</td>
<td>1:25</td>
<td>4°C, overnight</td>
</tr>
<tr>
<td>Cyp 2B</td>
<td>goat anti-rat</td>
<td>1:50</td>
<td>4°C, overnight</td>
</tr>
<tr>
<td>mEH</td>
<td>goat anti-rabbit</td>
<td>1:50</td>
<td>4°C, overnight</td>
</tr>
</tbody>
</table>

Table 2.3: Primary Antibody Incubation Conditions for Confocal Microscopy.
<table>
<thead>
<tr>
<th>Metabolic Enzyme</th>
<th>Origin of Antibody</th>
<th>Dilution of Antibody</th>
<th>Incubation Conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 2E1</td>
<td>rabbit anti-goat</td>
<td>1:75</td>
<td>37°C, 60 min</td>
</tr>
<tr>
<td>Cyp 2A</td>
<td>goat anti-rabbit</td>
<td>1:75</td>
<td>37°C, 60 min</td>
</tr>
<tr>
<td>Cyp 2B</td>
<td>rabbit anti-goat</td>
<td>1:75</td>
<td>37°C, 60 min</td>
</tr>
<tr>
<td>mEH</td>
<td>horse anti-goat</td>
<td>1:75</td>
<td>37°C, 60 min</td>
</tr>
</tbody>
</table>

Table 2.4: **Secondary Antibody Incubation Conditions for Confocal Microscopy**.

Sections were treated with Ribonuclease A (100 μg/ml) for 1 h, followed by staining for DNA with YOYO-1 (10 min; 5 nM). Slides were repeatedly rinsed with phosphate buffered saline (PBS), cover-slipped, and stored in the dark (4°C) until visualization. Immunofluorescence was visualized on a Leica confocal microscope with a xenon light source and the intensity was determined via an argon-krypton laser projected through the tissue into a photo-multiplier tube at $\lambda = 488$ and 647 nm for YOYO-1 (green) and Cy-5 (red), respectively. Images were captured at either 20X or 40X magnification. No autofluorescence was seen in unstained, coverslipped ovarian sections at $\lambda = 647$ nm.

Relative densitometric analysis using Scion Image Software (National Institutes of Health, Bethesda, MD) was utilized to compare relative intensities of staining. Background staining intensity was subtracted from each field. Multiple readings were taken throughout the sections. Analysis was performed at controlled settings on the confocal microscope in which 30-35 primordial follicles, 10-20 small primary follicles, 5-10 large primary follicles, 2-5 antral follicles, and 8-12 interstitial compartments were evaluated per group. Because the intensity of staining was very high for the interstitial cells at the settings in which the follicles were evaluated, these settings were changed to...
allow more accurate assessment of staining intensity in this ovarian fraction. However, all of the analyses for the interstitial cells were also compared at the same settings. Samples were normalized to control so multiple experiments could be compared.

**Functional Protein Isolation:** Total protein was extracted from either whole ovaries (obtained from either mice or rats) or isolated ovarian fractions (mice). Briefly, ovaries were removed and the oviduct and excess fat were trimmed away. The tissue (whole ovaries or isolated ovarian fractions) was lysed (buffer containing Triton-X, HEPES, NaCl, glycerol, SDS, EDTA, NaF, PMSF, leupeptin, and aprotinin) and homogenized. Samples were incubated on ice for 30 min and centrifuged (16,000 × g; 10 min). The resulting supernatant was collected and the protein concentration was determined utilizing the BCA Protein Assay® kit (λ=570 nm).

**Liver Microsome Preparation:** Following euthanization, livers from mice were excised. Microsomes were prepared according to Guengerich (1989). Briefly, livers from 4 mice/group were pooled and 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 (2,500 × g, 20 min; 9,000 × g, 1 h; 100,000 × g, 1 h). The microsomal fraction was collected and the protein concentration was determined utilizing the BCA Protein Assay® kit (λ=570 nm).
**Cyp 2E1 Activity Assay:** Cyp 2E1 catalyzes the hydroxylation of p-nitrophenol to p-nitrocatechol (Patten et al., 1992). In our studies, protein (200 µg) from whole ovarian homogenates was incubated (37° C, shaking water bath) in assay buffer (100 mM potassium phosphate, pH 6.8) containing substrate, (p-nitrophenol; 5 mM), and cofactor (NADPH; 20 mM) for 1 h (Chang et al., 1998a; Tierney et al., 1996). The final volume of the in vitro reaction was 250 µl. Following incubation, the reaction was stopped with the addition of 20% trichloroacetic acid 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, 2 M NaOH was added to each sample or standard (p-nitrocatechol; 10 nM-40 µM). Absorbance was measured at λ=520 nm on a 96-well plate reader (Molecular Devices) equipped with Soft Max Pro Software. 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 experiment was also performed in liver microsomes (data not shown). Optimal conditions for p-nitrophenol hydroxylation in whole ovaries utilized 200 µg of protein incubated for 1 h.

**Cyp 2A Activity Assay:** Cyp 2A catalyzes the hydroxylation of coumarin to 7-hydroxycoumarin (Waxman et al., 1991). In our studies, protein (200 µg) from whole ovarian homogenates was incubated (37° C, shaking water bath) in assay buffer (50 mM potassium phosphate, pH 7.4; 3 mM MgCl₂; 1 mM EDTA) containing substrate,
(coumarin: 50 μM), and cofactor (NADPH: 9 mM) for 1 h (Waxman and Chang, 1998; Pearce et al., 1992). The final volume of the in vitro reaction was 200 μl. Following incubation, the reaction was stopped with the addition of 2 N hydrochloric acid and samples were placed on ice. The samples were extracted with dichloromethane and concentrated (~ 50 μl). The samples were back extracted with sodium borate (30 mM) and the aqueous layer was transferred to a 96-well plate. Fluorescence (excitation λ = 410 nm; emission λ = 510 nm) was measured for standards (7-hydroxycoumarin: 1 nm – 10 μM) and samples on a fluorescent plate reader (Spectra Max Gemini XR) equipped with Soft Max Pro Software. 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 (30 min - 2 h) and protein concentration (50 - 1000 μg). To verify assay measurements, the experiment was also performed in liver microsomes (data not shown).

**Cyp 2B Activity Assay**: At low concentrations of substrate (~ 5 μM), Cyp 2B 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). In our studies, protein (200 μg) from whole ovarian homogenates was incubated (37° C, shaking water bath) in assay buffer (100 mM potassium phosphate, pH 7.4; 3.3 mM MgCl2) containing substrate, (EFC: 5 μM), and cofactor (NADPH: 12 mM) for 1 h (Chang et al., 1998b; Code et al., 1997). The final volume of the in vitro reaction was
200 µl. Following incubation, the reaction was stopped with the addition of 2 N hydrochloric acid 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 (HFC: 1 nM - 10 µM). Fluorescence (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. 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 experiment was also performed in liver microsomes (data not shown). Optimal conditions for EFC metabolism in whole ovaries utilized 200 µg of protein incubated for 1 h.

**mEH Activity Assay:** Protein (25 µg) from isolated ovarian fractions (F1, F2, F3, Int) was incubated (37°C, shaking water bath) in Tris buffer (100 mM, pH 9.0) containing 1 µl [³H]-cis-stilbene oxide (CSO) for 2 h. A 5 mM stock solution of substrate ([³H]-CSO) in ethanol was utilized. The [³H]-CSO was over 99% pure (purified by preparative TLC) and the specific activity was 1 mCi/mmol. The [³H]-CSO added to the reaction was prediluted (1:2) in non-radiolabeled CSO (5 mM in ethanol) to yield approximately 15,000 cpm/1 µl. The final substrate concentration was 12.5 µM. The final volume of the *in vitro* reaction was 200 µl. Ethanol made up 0.5% of the total reaction volume.
Following incubation, the reaction was stopped with the addition of 200 µl 2,2,4-
trimethylpentane. The samples were vortexed and centrifuged, resulting in a phase
separation. By this approach, unreacted CSO partitions into the organic phase, while
reacted CSO product (diol) partitions into the aqueous phase. An aliquot (150 µl) of the
aqueous phase was removed and the reaction product (diol) was counted by standard
scintillation counting. Additionally, experiments were performed to ensure linearity of
the reaction in terms of incubation time (30 min - 3 h) and protein concentration (10 - 50
µg). Optimal conditions for CSO metabolism in ovarian fractions utilized 25 µg of
protein incubated for 2 h. After the incubation, the average percent of the substrate
consumed was 4%.

Ovarian Culture: B6C3F1 mouse pups (d 4) were killed by CO₂ inhalation and then
decapitated. The entire female reproductive tract (ovary, oviduct, and uterus) was
surgically removed with aid of a dissecting microscope and fine tipped forceps in a sterile
environment and placed into ice cold Hank’s buffered saline (without CaCl₂, MgCl₂, or
MgSO₄). Excess fat, connective tissue, oviduct, and uterus were trimmed away. Ovaries
were floated on membranes (cut out from Millicell-CM filter inserts) atop of media
(Ham’s F-12/DMEM containing 1 mg/ml BSA, 1 mg/ml albumax, 50 µg/ml ascorbic
acid, 27.5 µl/ml transferrin, 5 U/ml penicillin, and 5 U/ml streptomycin; 500 µl) with the
appropriate treatment in 4-well culture plates. A small amount of medium was placed on
top of the ovary to prevent the tissue from drying out and to optimize exchange of
nutrients and oxygen (Figure 2.2). One or two ovaries were cultured in each well. Plates
Figure 2.2: *In vitro* culture system set-up utilizing whole ovaries. Ovaries from 4 d old mice were placed on membranes that were floated on top of 500 µl medium containing the appropriate treatment group. A small amount of medium was placed on top of the ovary to prevent the tissue from drying out and to optimize exchange of nutrients and oxygen.
were incubated in a humidified environment (95% air. 5% CO₂) for 15 days. Medium and treatments (vehicle control, 500 μM 1.2-VCM, 1000 μM 1.2-VCM, or 30 μM VCD) were changed every other day. Following 15 days of *in vitro* incubation, the ovaries were fixed in Bouin's fixative for histology.

**Ovarian Follicle Counts:** The fixed ovaries were embedded in paraffin, serially sectioned (5-7 μm thick), and stained with hematoxylin and eosin for histological evaluation at the light microscope level. The numbers of follicles in each stage of development (primordial, small primary, large primary) were evaluated in every 6th section. This analysis was previously characterized by Devine *et al* (2002). Only those follicles with distinct nuclei were included in the counts. Primordial follicles were classified as having a single layer of squamous granulosa cells. Primary follicles were classified when the granulosa cells were more cuboidal or rounded in shape. Small primary follicles contained less than 20 granulosa cells, whereas large primary follicles contained more than 20 granulosa cells. All follicle numbers were obtained from blinded counting.

**Statistical Analyses:** Comparisons 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. The assigned level of significance for all tests was *p*<0.05.
CHAPTER 3

EXPRESSION AND ACTIVITY OF CYTOCHROMES P450 2E1, 2A, AND 2B IN THE MOUSE OVARY: THE EFFECT OF 4-VINYLCYCLOHEXENE AND ITS DIEPOXIDE METABOLITE

INTRODUCTION:

The cytochromes P450 (CYP 450) belong to a superfamily of enzymes involved in various phase I metabolic reactions, including hydrolysis, reduction, and oxidation. These reactions are critical in determining the fate of numerous xenobiotics, including many drugs, environmental contaminants, as well as endogenous substrates, such as steroids, prostaglandins, fatty acids, and retinoids (Porter and Coon, 1991; Gonzales, 1989). CYP 450-mediated metabolism can produce water soluble metabolites, which facilitate excretion of the compound from the body. Additionally, the metabolism can form electrophilic metabolites that are highly reactive and result in toxic, and possibly carcinogenic injury (Wrighton and Stevens, 1992). Thus, depending upon the nature of the compound, CYP 450 may be responsible for either detoxification or bioactivation reactions.

Numerous CYP450 isoforms have been characterized in a variety of tissues. These enzymes are classified by their structural homology, overlapping substrate specificities, and induction and/or inhibition potentials (Nebert and Gonzales, 1987; Gonzales, 1989). As expected, the liver contains the highest concentration of CYP450
enzymes. However, many extrahepatic tissues, such as kidney, lung, brain, skin, testis, and ovary, to name a few, express CYP 450 and are involved in xenobiotic metabolism (Krishna and Klotz, 1994; Mukhtar et al., 1978). This can be beneficial or detrimental in terms of toxic insult to a particular organ. In the ovary, for instance, Shiromizu and Mattison (1984) evaluated the effect of unilateral intraovarian (i.o.) injection of benzo(a)pyrene, B(a)P, on the number of primordial oocytes in mice. B(a)P, which must be bioactivated to the toxic metabolite, benzo(a)pyrene-7,8-dihydrodiol-9,10-epoxide, only destroyed follicles in those ovaries injected with B(a)P. Furthermore, follicle loss was prevented when the potent CYP 450 inhibitor, \( \alpha \)-naphthoflavone, was administered (i.p.) concurrently with B(a)P (i.o.). This study supports the hypothesis that the ovary contains CYP 450 dependent monoxygenases and that the ovary can metabolize xenobiotics. Additional studies by Bengtsson et al. (1983, 1987, 1992) have also provided evidence of ovarian metabolism of some of the polycyclic aromatic hydrocarbons by ovarian granulosa cells. However, it is not known which CYP 450 isoforms are present in the ovary or in what sizes of follicles.

4-Vinylcyclohexene (VCH) is an industrial chemical used in the manufacture of flame retardants, insecticides, and plasticizers, that causes ovarian toxicity in mice, but not rats. However, the VCH-monoepoxides and the diepoxide metabolite (VCD) are ovotoxic in both species (Smith et al., 1990a; Smith et al., 1990b). Thus, the mouse is more sensitive to the effects of VCH than the rat. This species specific response may be explained by differences in metabolism in which the mouse may exhibit a greater ability to bioactivate VCH (via CYP 450) and a lesser ability to detoxify VCD (via microsomal
epoxide hydrolase, mEH; Figure 1.3). Structure activity studies verified that VCD is the ultimate ovotoxic metabolite responsible for destruction of immature, small pre-antral follicles (Doerr et al., 1995; Smith et al., 1990b). Loss of follicles caused by VCH or VCD is selective for small pre-antral follicles (primordial and primary). This is relevant because the ovary contains a finite number of follicles, which once depleted cannot be regenerated. Destruction of this population of follicles, therefore, may represent a risk for early menopause in exposed women (Hooser et al., 1994).

The exact mechanism of VCH induced-ovotoxicity in mice is not known. However, metabolism (bioactivation and detoxification) is likely a key component. The hepatic CYP 450 isoforms thought to be involved in the bioactivation of VCH to VCD in mice include Cyp 2A, Cyp 2B, and Cyp 2E1 (Smith et al., 1990c; Doerr-Stevens et al., 1999). Studies by Fontaine et al. (2001a, 2001b) showed that daily dosing with VCH induced hepatic protein levels and activities of Cyp 2A and Cyp 2B in mice, but not rats. Cyp 2E1 was not induced in mice or rats following in vivo dosing with VCH. In hepatic microsomal incubations from Cyp 2E1 deficient and wild type mice, VCH could be metabolized to similar amounts of VCH 1,2-monoepoxide and VCH 7,8-monoepoxide, suggesting that Cyp 2E1 is not required for epoxidation of VCH in the liver.

Currently, it is not known whether the ovary participates in VCH-related metabolism in mice. 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, in vivo exposure to VCH may present a different picture. Studies in our laboratory demonstrated that the ovary has the
capacity to be involved in detoxification reactions in both mice and rats. Our studies in mice showed that VCH or VCD dosing alters expression of mRNA, distribution of total protein, as well as functional activity for mEH in different sizes of ovarian follicles (Cannady et al., 2002). Flaws et al (1994b) showed that pre-antral follicles isolated from rats can detoxify VCD to the non-toxic tetrol metabolite (4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane). The capacity for bioactivation of VCH in the ovary is not known, nor is the compartmentalization of the CYP 450 enzymes/isoforms within different sizes of ovarian follicles representing different stages of development. Because VCD specifically destroys small pre-antral (primordial and primary) follicles, ovarian distribution of CYP 450 may impact susceptibility to exposure within specific follicle populations. Thus, this study was designed to investigate ovarian Cyp 450 isoforms (Cyp 2E1, Cyp 2A, and Cyp 2B) 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 Cyp 450 isoforms.

**EXPERIMENTAL METHODS:**

Female B6C3F1 mice (d 28) were utilized for all studies. Animals were dosed daily (15 d) with either vehicle-control, VCH, or VCD. Following euthanasia, ovaries were removed and distinct compartments of follicles and interstitial cells were isolated. Total RNA was recovered from the tissue and reverse transcribed into cDNA. cDNA was amplified by realtime RT-PCR. Total protein distribution was evaluated by confocal
microscopy. Additionally, protein from whole ovarian homogenates was utilized for Cyp 450 catalytic assays. All methods are discussed in detail in Chapter 2.

RESULTS:

Realtime RT-PCR: mRNA encoding various cytochromes P450 was evaluated in follicles isolated from mouse ovaries following repeated, in vivo exposure (15 daily doses) with vehicle, VCH, or VCD. mRNA encoding Cyp 2E1, Cyp 2A, and Cyp 2B was detected in all ovarian fractions that were studied (F1, small pre-antral, 25-100 μm; F2, large pre-antral, 100-250 μm; F3, antral, > 250 μm; Int) from all treatment groups. Expression levels for mRNA encoding the CYP 450 isoforms were compared in each ovarian fraction from vehicle-treated animals (Table 3.1).

Table 3.1

<table>
<thead>
<tr>
<th>Cyp 450 Isoform</th>
<th>F1</th>
<th>F2</th>
<th>F3</th>
<th>Int</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp 2E1</td>
<td>0.005±0.001 mRNA:18 S rRNA</td>
<td>0.776±0.55 mRNA:18 S rRNA</td>
<td>0.774±0.45 mRNA:18 S rRNA</td>
<td>0.324±0.16 mRNA:18 S rRNA</td>
</tr>
<tr>
<td>Cyp 2A</td>
<td>0.027±0.03 mRNA:18 S rRNA</td>
<td>0.044±0.02 mRNA:18 S rRNA</td>
<td>0.680±0.6 mRNA:18 S rRNA</td>
<td>2.082±0.6 mRNA:18 S rRNA *</td>
</tr>
<tr>
<td>Cyp 2B</td>
<td>0.133±0.06 mRNA:18 S rRNA</td>
<td>0.506±0.43 mRNA:18 S rRNA</td>
<td>0.600±0.46 mRNA:18 S rRNA</td>
<td>2.903±0.12 mRNA:18 S rRNA *</td>
</tr>
</tbody>
</table>

Table 3.1: mRNA expression of Cyp 450 isoforms in distinct ovarian fractions from untreated mice. Arbitrary values expressed as a ratio of mRNA:18S rRNA. ( * statistically significant from Cyp 2E1: p<0.05)
Cyp 2B was most highly expressed in F1 follicles, compared to other Cyp 450 isoforms. whereas Cyp 2E1 and Cyp 2B were both highly expressed in F2 follicles. All three isoforms were abundant in F3 follicles. Int cells expressed significantly more \((p<0.05)\) Cyp 2A and Cyp 2B, compared to Cyp 2E1. Repeated daily dosing \((15\, \text{d})\) with VCH or VCD altered gene expression of the isozymes evaluated. VCH and VCD dosing increased \((p<0.05)\) mRNA encoding Cyp 2E1 in F1 follicles \((645\pm14\%\) above control, VCH; \(582\pm16\%\) above control, VCD) and F3 follicles \((168\pm7\%\) above control, VCH). compared to control, but had little effect in the other ovarian fractions (Figure 3.1A).

Following repeated dosing with VCH or VCD, mRNA encoding Cyp 2A was increased \((p<0.05)\) in F1 follicles \((689\pm8\%\) above control, VCH; \(730\pm22\%\) above control, VCD) and Int cells \((207\pm19\%\) above control, VCH), but dosing had little effect in F2 and F3 follicles (Figure 3.1B). mRNA encoding Cyp 2B was increased \((p<0.05)\) only in F1 follicles following VCH dosing \((246\pm7\%\) above control), in the other ovarian fractions, mRNA was not altered by dosing (Figure 3.1C).
Figure 3.1: Semi-Quantitative RT-PCR for Cyp 2E1, Cyp 2A, and Cyp 2B. Female B6C3F1 mice (d 28) were given repeated daily doses (15 d; ip) of sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). 4 h following the final dose, ovaries were collected and follicle fractions were prepared. Total RNA was isolated from ovarian compartments and reverse transcribed into cDNA using random primers. Realtime RT-PCR was utilized for semi-quantification of mRNA encoding Cyp 2E1, Cyp 2A, and Cyp 2B, as described in methods. 18S rRNA, was determined to correct for sample variation. Final values for mRNA were calculated as a ratio of Cyp 450 mRNA:18S rRNA (treatment/control; Tx/Con). A) Cyp 2E1 expression. B) Cyp 2A expression. C) Cyp 2B expression. (n=4-6; p<0.05; * significantly different from control).
Confocal Microscopy: Total protein for Cyp 2E1, Cyp 2A, and Cyp 2B was visualized by immunostaining in the mouse ovary. Protein 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 CYP 450 protein isoforms. As measured by densitometry, a reduction (p<0.05) in Cyp 2E1 protein was observed in Int cells (19±2% below control, VCH). Staining intensity for Cyp 2E1 was not altered in other follicle populations (Figure 3.2A,B). VCH dosing caused an increase (p<0.05) in staining intensity for Cyp 2A protein, also in the Int cells (39±5% above control, VCH). No effects of VCH or VCD dosing were observed in other sizes of ovarian follicles (Figure 3.2C,D). Repeated dosing also altered the staining intensity for Cyp 2B. Dosing with VCH caused an increase (p<0.05) in Cyp 2B staining intensity in granulosa cells of small primary follicles (F1; 187±42% above control). Increased staining intensity was also observed in granulosa cells of antral follicles (F3) following dosing (63±8% above control. VCH; 45±8% above control. VCD; Figure 3.2E,F,G).

Cyp 2E1 Activity Assay: Specific activity for Cyp 2E1 was analyzed by hydroxylation of p-nitrophenol in whole ovarian homogenates from mice following in vivo dosing (15 daily doses) with vehicle, VCH, or VCD. Basal levels of Cyp 2E1 activity were measured in ovaries from vehicle-treated mice (0.0726±0.015 nmol/min/mg; Figure 3.3). Repeated dosing with VCH induced (p<0.05) activity of Cyp 2E1 (0.181±0.022 nmol/min/mg; Figure 3.4). Dosing with VCD did not affect Cyp 2E1 activity (0.126±0.020 nmol/min/mg; Figure 3.4).
Figure 3.2: Ovarian distribution of Cyp 2E1, Cyp 2A, and Cyp 2B in ovarian follicles and interstitial cells by confocal microscopy. B6C3F1 mice (d 28) were dosed daily (15 d: ip) with sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d).

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 Cyp 450 isoform. Cyp 2E1 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). Cyp 2A 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). Cyp 2B 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 granulosa cells from small primary follicles was significantly increased 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 animals/group)
Figure 3.2

A

B

C

D

Cyp 2E1 - Con

Cyp 2E1 - VCH

Cyp 2A - Con

Cyp 2A - VCH
Figure 3.2 - Continued

Cyp 2B - Con

Cyp 2B - VCH

Immunonegative
Figure 3.3: Unstimulated Cyp 450 activity in whole ovaries obtained from mice. Ovaries were collected and homogenates were prepared from vehicle-treated mice (d 42). Total protein was isolated from ovarian homogenates. Protein (200 μg) was incubated with p-nitrophenol, coumarin, or 7-ethoxy-4-trifluoromethyl coumarin for 1 h. The product was concentrated and absorbance (Cyp 2E1: λ=520 nm) or fluorescence (Cyp 2A. Cyp 2B: λex=425 nm, λem=525 nm) was measured. Values were determined by linear regression and are expressed as nmol/min/mg of protein. (n=4-7; p<0.05; * significantly different from Cyp 2B; ND= not detectable)
Figure 3.4: Effect of VCH/VCD dosing on Cyp 2E1 activity in whole ovaries from mice. Female B6C3F1 mice (d 28) were dosed daily (15 d; ip) with sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). 4 h following the final dose, ovaries were collected, and protein prepared, as described in methods. Protein (200 µg) was incubated with p-nitrophenol for 1 h. Samples were processed as presented in Figure 3.3. Values are expressed as nmol/min/mg of protein. (n=4; p<0.05; * significantly different from control)
**Cyp 2A Activity Assay:** No specific activity for Cyp 2A could be measured in ovarian homogenates from any treatment group, although linearity of hydroxylation of coumarin to 7-hydroxycoumarin could be measured in liver microsomes.

**Cyp 2B Activity Assay:** Specific activity for Cyp 2B was evaluated by O-deethylation of 7-ethoxy-4-trifluoromethyl coumarin in whole ovarian homogenates obtained from mice dosed daily (15 d) with vehicle control, VCH, or VCD. Basal levels of Cyp 2B activity in vehicle-treated ovaries were $0.0037 \pm 0.001$ nmol/min/mg (Figure 3.3). Furthermore, dosing did not affect Cyp 2B activity ($0.0036 \pm 0.001$ nmol/min/mg, VCH; $0.0034 \pm 0.001$ nmol/min/mg, VCD; Figure 3.5).
Figure 3.5: Effect of VCH/VCD dosing on Cyp 2B activity in whole ovaries from mice. Female B6C3F1 mice (d 28) were dosed daily (15 d; ip) with sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). 4 h following the final dose, ovaries were collected, and protein prepared, as described in methods. Protein (200 µg) was incubated with 7-ethoxy-4-trifluoromethyl coumarin for 1 h. Samples were processed as presented in Figure 3.3. Values are expressed as nmol/min/mg of protein. (n=7)
DISCUSSION:

The mammalian ovary is a heterogeneous mixture of diverse structures. It contains follicles in different stages of maturity, from the smallest, primordial, to the most mature, large antral follicles. Our studies have demonstrated that various Cyp 450 enzymes (Cyp 2E1, Cyp 2A, and Cyp 2B), important in a variety of metabolic reactions, are expressed in the mouse ovary. Cyp 450 protein is distributed throughout the ovary, being expressed in all ovarian cell types including oocytes, granulosa cells, theca cells, and interstitial cells. Interestingly, the highest mRNA expression levels and staining intensity for all isoforms evaluated was in the interstitial cells, compared to other ovarian fractions. Additionally, Cyp 2E1 and Cyp 2B are functional, as evaluated by metabolism of model substrates. Due to assay sensitivity, enzyme activities were made in whole ovarian homogenates instead of distinct ovarian fractions.

Repeated in vivo dosing with VCH or VCD altered Cyp 450 expression. A significant increase in expression of mRNA encoding Cyp 2E1 was observed in F1 follicles, those follicles specifically targeted by VCD, as well as non-targeted F3 follicles after VCH or VCD dosing, compared to vehicle-treated animals. Although total protein for Cyp 2E1 was slightly decreased in Int cells following VCH-treatment, whole ovarian Cyp 2E1 activity was increased. Despite the decrease in total protein, the increase in Cyp 2E1 specific activity is not alarming because of the very high levels of Cyp 2E1 total protein in the Int cells. Additionally, enzyme activity studies 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 confocal data, any size of follicle could potentially convert the model substrate, p-nitrophenol. However, due to the abundant protein distribution in the Int cells, this ovarian compartment likely contributes significantly to this metabolism.

A significant increase in expression of mRNA encoding Cyp 2A was observed in the targeted F1 follicles following VCH, as well as VCD, dosing. VCH dosing also increased expression of Cyp 2A in the Int cells. Total Cyp 2A protein was also significantly induced in the Int cells, thus correlating with the mRNA data. However, these changes in mRNA levels and total protein did not correlate with functional protein, as specific activity for Cyp 2A was not reproducibly detected in any treatment group.

mRNA encoding Cyp 2B was increased in F1 follicles following repeated dosing with VCH. VCH dosing increased total protein for Cyp 2B in granulosa cells of small primary (F1) and antral (F3) follicles. Furthermore, VCD dosing also increased protein in F3 follicles. Although Cyp 2B could be measured, its activity was not significantly altered by VCH or VCD dosing. However, based on the distribution of total protein, the functional ovarian compartment is likely to be Int cells and possibly antral follicles (due to their high abundance of granulosa cells).

Our studies evaluated mRNA expression, total protein, and functional activity following 15 d of repeated daily dosing. Thus, the effect of dosing on Cyp 2E1, Cyp 2A, and Cyp 2B expression profiles are not known at earlier or later time points. However, our studies suggest that Cyp 2E1 is likely the major contributor to ovarian metabolism of VCH, as baseline activity for its model substrate was significantly higher than the other Cyp 450 isoforms evaluated. Interestingly, Cyp 2E1 did not appear to be involved in the
hepatic bioactivation of VCH at this same time point (Fontaine et al., 2001b). Rather, studies by Fontaine et al. (2001a) showed hepatic induction of total and functional protein for Cyp 2A and Cyp 2B following 10 d of dosing in the mouse. In the ovary, temporal effects on Cyp 2A and Cyp 2B expression may be different from the liver. Conversely, a different isoform profile may predominate in the ovary as compared to the liver.

Our studies showed that mRNA encoding Cyp 2E1, Cyp 2A, and Cyp 2B 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 Cyp 450 isoform proteins localized in the Int compartment. These cells are in the highly vascularized region of the ovary, thus the Int cells would likely be exposed to toxicants traveling through the blood stream. This metabolic potential may serve as a generalized functional role for the interstitium, whose physiological function has remained largely unclear. Furthermore, this ovarian compartment may be involved in either mono-epoxidation of VCH and/or subsequent epoxidation of the monoepoxides to VCD.

Although the liver is the primary organ of xenobiotic metabolism, extrahepatic tissues, including the ovary, may also be important tissue specific sites 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 \textit{in vitro} with the S9 fraction isolated from ovaries collected from mice dosed \textit{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 Bengtsson et al. (1983, 1987, 1992) have also demonstrated ovarian metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) and 3-MC in rats. Because metabolites of these compounds are known to be ovotoxic, ovarian metabolism is likely to play a role in bioactivating polycyclic aromatic hydrocarbons.

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 capacity (mRNA and functional protein) for bioactivation of VCH and/or the monoepoxides. Based on the basal and VCH-induced levels of Cyp 450 activity in whole ovaries, Cyp 2E1 is the likely isoform most involved in metabolic reactions, because activity was an order of magnitude higher compared to Cyp 2B activity, and Cyp 2A activity could not be reliably measured in mouse ovaries. The Int compartment may be the most important ovarian compartment involved in bioactivation of VCH, due to its large metabolic potential. Future studies will be aimed at determining more precisely the extent to which the ovary contributes to ovotoxicity induced in mice by VCH. This information can serve to better elucidate the metabolic role of the ovary in responding to xenobiotic exposures in general.
INTRODUCTION:

Epoxide hydrolase is an important metabolic enzyme that catalyzes the addition of water to alkene epoxides and arene oxides. The five characterized classes of mammalian epoxide hydrolase include three cytosolic forms (hepoxilin A₃, leukotriene A₄, soluble) and two microsomal forms (cholesterol 5,6-oxide, microsomal). Both the soluble and microsomal forms of epoxide hydrolase are involved in xenobiotic metabolism. Cytosolic epoxide hydrolase preferentially metabolizes trans-substituted epoxides, while microsomal epoxide hydrolase (mEH) metabolizes cis-substituted epoxides. Microsomal epoxide hydrolase has a wide substrate specificity and has the capacity to both bioactivate and detoxify xenobiotics (Omiecinski et al., 2000). Some xenobiotics metabolized by mEH include the epoxide metabolites of polycyclic aromatic hydrocarbons, 1,3-butadiene, benzene, aflatoxin B₁ and the anticonvulsant drugs, phenytoin and carbamazepine (Fanucchi et al., 2000; Fretland and Omiecinski, 2000). Microsomal epoxide hydrolase is most abundant in the liver, but has been found in all other tissues examined to date, including the ovary (Dannan and Guengerich, 1982; Mukhtar et al., 1978; Oesh et al., 1977).
Many xenobiotic compounds that are or can form reactive epoxide metabolites are detoxified by mEH, including the epoxides of 4-vinylcyclohexene (Figure 1.3). 4-Vinylcyclohexene (VCH), an industrial chemical used in the manufacture of flame retardants, insecticides, and plasticizers, causes ovarian toxicity in mice. Bioactivation of VCH by the cytochrome P450 enzymes to the diepoxide metabolite (VCD) is required to produce the ovarian-specific toxic effects, which involves targeting and destruction of immature, small pre-antral follicles (Doerr et al., 1995; Smith et al., 1990b). The resulting ovotoxicity is relevant because the ovary contains a finite number of follicles, which once depleted cannot be regenerated. Thus, widespread destruction of pre-antral ovarian follicles can lead to premature ovarian failure. Additionally, ovarian failure is associated with an increased incidence of ovarian neoplasms (Flaws et al., 1994a; NTP 1989).

The exact mechanism of VCH induced-ovotoxicity is not known. However, metabolism (bioactivation and detoxification) is a key component, and is thought to be at least partially responsible for a species specific response, in which VCH is ovotoxic in the mouse, but not the rat. The VCH-monoepoxides and VCD are ovotoxic in both species (Smith et al., 1990a; Smith et al., 1990b). Specifically, it is thought that the mouse may be more sensitive to the effects of VCH compared to the rat because of a greater ability to bioactivate VCH (via cytochrome P450). Previous studies have suggested that hepatic cytochrome P450 isoforms 2A, 2B, and 2E1 may be involved in the bioactivation of VCH to VCD in mice (Smith et al., 1990c; Doerr-Stevens et al., 1999). Species comparison studies showed that VCH treatment caused increased hepatic
protein levels and activities of CYP 2A and CYP 2B in mice, but not rats. Although studies in human hepatic "supersomes" showed that human CYP 2E1 and human CYP 2B6 were capable of catalyzing VCH epoxidation. CYP 2E1 was not induced in mice or rats following VCH treatment. Furthermore, the same amount of VCH 1,2-monoepoxide and VCH 7,8-monoepoxide were formed from VCH in hepatic microsomal incubations from CYP 2E1 knock-out and wild type mice, suggesting that CYP 2E1 is not involved in VCH epoxidation in the liver (Fontaine et al., 2001a; Fontaine et al., 2001b).

Additionally, relative to the rat, the mouse has a lesser ability to detoxify VCD (via mEH) to the non-toxic tetrol metabolite (4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane). Studies by Salyers et al. (1993) showed that 6 h after receiving a single dose of [14C]-VCD, rats excreted 70% of the dose in the urine, whereas mice only excreted 30% of the dose in the urine. Likewise, it has been reported that mice have lower levels of mEH activity compared to humans or rats (Kitteringham et al., 1996; Krause et al., 1997). This information collectively supports the greater susceptibility of mice, compared with rats, to these chemicals.

Currently, it is not known whether ovarian mEH is involved in VCD metabolism in the mouse, although it has been proposed as a detoxification pathway in rat in vitro studies (Flaws et al., 1994b). Additionally, it is not known if mEH is compartmentalized within different sizes of ovarian follicles representing different stages of development. Because VCD specifically destroys small pre-antral (primordial and primary) follicles, ovarian distribution of mEH may influence either protection or destruction of specific follicle populations. Thus, the purposes of our studies were to 1) determine expression of
mRNA encoding for mEH in follicles isolated from mouse ovaries. 2) determine catalytic activity of mEH in follicles isolated from mouse ovaries. 3) identify ovarian distribution of mEH protein. and 4) determine the effects of *in vivo* dosing with VCH/VCD on mEH expression, activity and protein distribution.

**EXPERIMENTAL METHODS:**

Female B6C3F1 mice (d28) were utilized for all studies. Animals received either a single dose or 15 daily doses of vehicle-control, VCH, or VCD. Following euthanasia, ovaries were removed and distinct compartments of follicles and interstitial cells were isolated. Total RNA was recovered from the tissue and reverse transcribed into cDNA. cDNA was amplified by realtime RT-PCR. Total protein distribution was evaluated by confocal microscopy. Additionally, protein was isolated from the distinct ovarian compartments and mEH activity measured. All methods are discussed in detail in Chapter 2.

**RESULTS:**

**Realtime RT-PCR:** mRNA encoding mEH was evaluated in follicles isolated from mouse ovaries following *in vivo* pre-treatment (1 single dose or 15 daily doses) with vehicle, VCH, or VCD. mRNA encoding mEH was detected in tissue obtained from all treatment groups. mEH mRNA was expressed in all follicle populations that were studied (F1, small pre-antral, 25-100 μm; F2, large pre-antral, 100-250 μm; F3, antral, >250 μm) in the ovary, as well as in the Int cells. Expression levels for mRNA encoding
mEH were compared in different ovarian fractions from vehicle-treated animals, in which mature F3 follicles (13.52±5.9 mEH mRNA:18S rRNA) expressed significantly more mRNA encoding mEH compared to the other fractions (F1, 4.01±1.4 mEH mRNA:18S rRNA; F2, 3.06±1.1 mEH mRNA:18S rRNA; Int, 2.38±1.3 mEH mRNA:18S rRNA; p<0.05). There were no significant effects on gene expression following a single dose of VCH/VCD (data not shown). In contrast, repeated daily dosing (15 d) with VCH increased (p<0.05) expression of mRNA encoding mEH by 410±5% in F1 and 1379±4% in F2, compared to control (Figure 4.1). Conversely, VCH decreased (p<0.05) mEH expression by 60±27% in Int cells compared to control. Repeated dosing with VCD increased (p<0.05) mEH expression by 292±5% in F1, 381±11% in F2, and 153±7% in F3, whereas VCD did not affect mEH expression in Int cells. (Figure 4.1)
Figure 4.1: Relative semi-quantitative RT-PCR for mEH. Female B6C3F1 mice (d 28) were given repeated daily doses (15 d, ip) of sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). 4 h following the final dose, ovaries were collected and follicle fractions were prepared. Total RNA was isolated from ovarian follicle fractions and reverse transcribed into cDNA using random primers. Realtime RT-PCR was utilized for semi-quantitation of mRNA encoding mEH. A standard curve was generated from 1:5 serial dilutions of purified mEH PCR product. An aliquot of each dilution was used as PCR template to generate a standard curve. Arbitrary numbers were assigned to each standard and experimental samples were calculated from the standard curve. The housekeeping gene, 18S rRNA, was also determined for all samples as described for mEH. Final values for mEH expression were calculated as a ratio of mEH mRNA:18S rRNA (treatment/control: Tx/Con). (n=4-6; p<0.05; * significantly different from control; "a" and "b" significantly different from one another)
mEH Activity Assay: Microsomal epoxide hydrolase activity was analyzed in isolated mouse ovarian follicles following in vivo dosing (1 single dose or 15 daily doses) with vehicle, VCH, or VCD. Activity was detected in all groups examined. Levels of mEH activity in d 28 vehicle-treated animals were 56.7 ± 14.5 nmol/min/mg of protein in F1 follicles, 128.7 ± 65.6 nmol/min/mg of protein in F2 follicles, 61.7 ± 30.6 nmol/min/mg of protein in F3 follicles, and 85.8 ± 26.4 nmol/min/mg of protein in Int cells. In d 42 vehicle-treated mice, mEH activity was 34.2 ± 15.5 nmol/min/mg, 147.7 ± 42.1 nmol/min/mg, 19.3 ± 9 nmol/min/mg, and 16.3 ± 6.8 nmol/min/mg of protein in F1, F2, F3, and Int cells, respectively. Interestingly, the VCH/VCD non-target population of F2 follicles displayed significantly higher activity (p<0.05) compared to other fractions in this group (Figure 4.2). There were no significant effects in enzyme activity following a single dose of VCH or VCD (data not shown). However, repeated dosing with VCH or VCD did alter mEH activity (Figure 4.3). In F1 follicles (those targeted by VCH and VCD) there was an increase (p<0.05) in mEH activity (381 ± 11% VCH, 384 ± 27% VCD) above control. There was no effect of dosing in F2 follicles. while non-significant trends for increased mEH activity were seen in large antral (F3) follicles and Int cells following VCH or VCD dosing (344 ± 15% VCH, F3: 304 ± 69% VCD, F3: 482 ± 40% VCH, Int: 285 ± 28% VCD, Int) compared to control.
Figure 4.2: Unstimulated mEH activity in follicles and interstitial cells isolated from mouse ovaries. Ovaries were collected and follicle fractions prepared from vehicle-treated mice (d 42). Total protein was isolated from ovarian follicle fractions. Protein (25 μg) was incubated with $[^3H]$-cis stilbene oxide for 2 h. 2,2,4-Trimethylpentane was added to stop the reaction and resulted in a phase separation. The unreacted cis-stilbene oxide partitions into the organic phase while the reaction product (diol) partitions into the aqueous phase. An aliquot of the aqueous phase was removed and the radioactivity counted by standard scintillation counting. Values are expressed as nmol/min/mg of protein. (n=4; p<0.05; * significantly different from control)
Figure 4.3: mEH activity in follicles and interstitial cells isolated from mouse ovaries. Female B6C3F₁ mice (d 28) were given repeated daily doses (15 d; ip) of sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). 4 h following the final dose, ovaries were collected, follicle fractions were prepared, and protein (25 μg) was incubated with [³H]-cis stilbene oxide for 2 h. Samples were processed as presented in Figure 4.2. Values are expressed as a ratio of treatment/control (Tx/Con). (n=4; p<0.05; * significantly different from control)
**Confocal Microscopy:** Total mEH protein was visualized in the mouse ovary. Protein was present in oocytes, granulosa cells, and theca cells, as well as the surrounding Int cells. Following repeated dosing, VCH and VCD increased mEH protein expression in theca cells of growing pre-antral follicles (F2), as measured by densitometry (56 ± 0.8% above control, VCH; 29 ± 0.9% above control, VCD: \( p<0.05 \)) compared to all other ovarian follicle components and Int cells in vehicle-treated mice (Figure 4.4). No autofluorescence was seen in unstained, coverslipped ovarian sections at \( \lambda = 647 \text{ nm} \).
Figure 4.4: mEH protein distribution in ovarian follicles and interstitial cells by confocal microscopy. B6C3F1 mice (d 28) were dosed daily (15 d; ip) with sesame oil (vehicle), VCH (7.4 mmol/kg/d), or VCD (0.57 mmol/kg/d). Ovaries were removed 4 h after the final dose, fixed in 4% formalin, sectioned (5 μm thick, every 7th section), and incubated overnight with a goat anti-rabbit mEH polyclonal antibody. Immunofluorescence for mEH protein was visualized on a Leica confocal microscope with a xenon light source and the intensity was determined via an argon-krypton laser projected through the tissue into a photo-multiplier tube at $\lambda = 488$ nm for YOYO-1 (green, DNA stain) and $\lambda = 647$ nm for Cy-5 (red; mEH). All images were captured at 40X magnification. Relative changes in staining intensity were made by densitometry using Scion Image Software. Background staining intensity was subtracted from each field. Multiple readings were taken throughout the sections. Analysis was performed on different sizes of ovarian follicles and interstitial cells at controlled settings on the confocal microscope. mEH protein was visualized in (A) Fl, F2, and Int from vehicle-treated mice. (B) Fl, F2, and Int from VCH-treated mice, in which the staining intensity in theca cells of growing follicles (F2) was significantly increased compared to control ($p<0.05$). and (C) Fl, F2, and Int from VCD-treated mice, in which the staining intensity in theca cells of growing follicles (F2) was significantly increased compared to control ($p<0.05$). (D) 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 animals/group)
Figure 4.4

A  F2

Int

Theca Cells

F1

mEH - Con

B  F2

Int

F1

Theca Cells

mEH - VCH

C  F2

Int

F1

Theca Cells

mEH - VCD

D

Immunonegative
DISCUSSION:

The ovary is a heterogeneous organ, containing follicles in various stages of maturity, from the smallest, primordial, to the most mature, large antral follicles. Our studies have demonstrated that mEH, an important metabolic enzyme, is expressed and is functional within these specific follicle types, as well as in interstitial cells. Additionally, confocal studies revealed distribution of mEH throughout the ovary, with protein being expressed in the oocytes, granulosa cells, and theca cells, as well as interstitial cells, where the highest staining intensity was observed. In ovaries from vehicle-treated animals, mRNA expression of mEH was greatest in antral follicles (F3), while the greatest functional activity was measured in growing, pre-antral follicles (F2).

Repeated in vivo dosing with VCH or VCD modulated expression of ovarian mEH. A significant increase in expression of mRNA encoding mEH was observed in F1 follicles (those targeted by VCD), as well as non-targeted F2 follicles after VCH or VCD-treatment compared to vehicle-treated animals. Furthermore, the effects of VCH treatment caused a significantly greater increase in mRNA encoding mEH compared to VCD in these same follicle populations. These differences in response to VCH/VCD dosing may be explained by 1) the higher dose of VCH (7.4 mmol/kg) compared to VCD (0.57 mmol/kg), resulting in a higher concentration of metabolites being formed and/or 2) the presence of the VCH monoepoxides (vinylcyclohexene 1,2-monoepoxide or vinylcyclohexene 7,8-monoepoxide), which are also substrates for mEH and can be metabolized to non-toxic diols (vinylcyclohexene 1,2-diol or vinylcyclohexene 7,8-diol).
mEH activity in F1 follicles was also induced following VCH/VCD dosing, thus correlating with the mRNA data. A correlation between mEH mRNA levels and protein has been previously noted (Cho and Kim, 1997; Cho and Kim, 2000; Kim et al., 1998). The correlation between mRNA and specific activity was not seen in F2 follicles. However, induction in F2 follicles may be unimportant because of the already high levels of enzyme activity in this follicle population, relative to the other ovarian fractions. Regardless of mechanism, it appears ovarian pre-antral follicles have acquired a protective response via mEH induction. However, after 12 d of repeated daily dosing, extensive follicle loss is evident in mice (Kao et al., 1999). Therefore, the detoxification system likely becomes overwhelmed, and vulnerability of this follicle population (F1) compared to the larger, more mature follicles may in part be the reason for their greater susceptibility to the ovotoxic effects of VCD.

After 15 d of dosing, only primordial and primary follicles (contained in F1) are impacted by VCH/VCD dosing (d1-d15; Springer et al., 1996; Kao et al., 1999). Dosing with VCH/VCD does not directly affect the population of follicles contained in F2 (large growing, pre-antral). However, following 30 d of dosing, there is a reduction in the number of large growing follicles (Flaws et al., 1994a). This has been interpreted to reflect the effect of a reduced population of primordial and primary follicles from which to recruit growing follicles. Likewise, ultimately F3 follicles will also be impacted as ovarian failure occurs (Hooser et al., 1994; Mayer et al., 2002). Although not directly targeted by VCH/VCD, other sizes of ovarian follicles, as well as the interstitial compartment, may be involved in the detoxification of VCH-monoepoxides and/or VCD.
thus protecting the vulnerable F1 follicle population from exposure to and destruction by VCD. For instance, F2 follicles from vehicle-treated animals have a significantly greater amount of mEH activity compared to other fractions. Additionally, there was a non-significant trend for mEH activity to be induced in F3 follicles and interstitial cells following repeated exposure to VCH/VCD. Although, as mentioned previously, any defense provided by these non-target populations is not sufficient to protect the ovary and prevent follicle destruction, as follicle loss was first seen in mice on d 12 of dosing (Kao et al., 1999).

Interestingly, expression of mEH protein was upregulated in theca cells of growing, pre-antral follicles (F2), as observed and quantified by confocal microscopy. Theca cells are highly vascularized, surround the follicle, and may serve as a protective cell barrier between blood borne toxic metabolites and the vulnerable oocyte. In contrast, primordial follicles (F1) do not have a developed theca cell layer. Thus, this extra barrier of protection is not in place and may be another factor contributing to the specificity of targeting in this population of follicles. It is thought that a subpopulation of interstitial cells is derived from theca cells of follicles undergoing atresia, a natural form of cell death within the ovary (Erickson et al., 1985). Thus, retaining detoxification capabilities as theca cells become interstitial cells would serve as a constant protective mechanism for the ovary. The apparently large metabolic potential of the interstitial cells suggests a possible physiologic and toxicologic role of this ovarian cell type.

Although the liver is the primary organ of xenobiotic metabolism, extrahepatic tissues, including the ovary, may also be important tissue specific sites of bioactivation
and detoxification. For example, Shiromizu et al. (1984) injected benzo(a)pyrene, B(a)P, directly into the ovary to evaluate the role of ovarian metabolism on B(a)P-induced ovotoxicity in mice. As a result, primordial follicles were destroyed, suggesting that the ovary has the capacity to metabolize B(a)P to the toxic diol-epoxide metabolite (7,8-dihydrodiol-9,10-epoxide) (Mattison et al., 1983). Studies by Bengtsson et al. (1992, 1987, 1983) demonstrated ovarian metabolism of 7,12-dimethylbenz(a)anthracene (DMBA) and 3-methylcholanthrene (3-MC) in rats. Since metabolites of these compounds are known to be ovotoxic, ovarian metabolism likely plays a role in activating polycyclic aromatic hydrocarbons. Studies by Flaws et al. (1994b) demonstrated a role for ovarian detoxification of epoxide metabolites, because follicles isolated from rat ovaries converted VCD to the non-toxic tetrol, presumably by mEH. The studies presented here further suggest a role for mEH in ovarian metabolism of epoxides by showing the capability for ovarian detoxification of both the mono- and diepoxide metabolites of VCH in mice. These findings are unique in that they have evaluated ovarian metabolic enzymes, notably mEH, in isolated ovarian compartments, whereas to date, previous studies have been performed in microsomes prepared from whole ovaries. Our approach allows investigation of differential responses within targeted and non-targeted subpopulations of ovarian tissue.

Recently, studies by Coller et al. (2001) and Hattori et al. (2000) evaluated mEH in the human ovary. By immunohistochemical analysis, Coller et al. showed that mEH is highly expressed throughout the ovary. Additionally, mEH was found to be highly expressed in ovarian tumors, including theca-fibromas. Studies by Hattori et al. (2000)
also evaluated mEH distribution throughout the ovary via immunohistochemistry. mEH was detected in granulosa cells and theca cells. In vitro studies utilizing the mEH inhibitor, 1,2-epoxy-3,3,3-trichloropropane, caused a dose-dependent decrease in the production of 17β-estradiol. Thus, mEH may also be involved in different ovarian steroidogenic pathways. Most importantly, however, these reported findings support that mEH is likely to play a role in human ovarian function.

mEH is expressed and is functional in follicles isolated from mouse ovaries. Furthermore, in vivo dosing with VCH or VCD affects both mEH mRNA levels and specific activity. Specifically, F1 follicles (those targeted by VCD) demonstrated increased expression of mRNA and greater enzyme activity, compared to other ovarian fractions following repeated dosing with VCH/VCD. Thus, after repeated exposure, F1 follicles may acquire a greater ability to participate in detoxification of VCD as a protective measure against follicle destruction. Future studies will investigate in vitro metabolism utilizing the VCH-monoepoxides and VCD as substrates to directly evaluate the role of the ovary in both bioactivation and detoxification.
CHAPTER 5

METABOLISM OF MODEL SUBSTRATES IN RAT OVARIIES: RESPONSES TO 4-VINYLCYCLOHEXENE AND EPOXIDE METABOLITES

INTRODUCTION:

Repeated exposure to VCH causes ovarian toxicity in the mouse, but not the rat. However, exposure to the epoxide metabolites (VCM and VCD) causes ovotoxicity in both the mouse and the rat. Thus, differences in metabolism, specifically the first epoxidation reaction of VCH, may partially explain the species specific responses to the chemical. In vivo and in vitro studies by Smith et al. (1990a), showed that mice form greater amounts of 1,2-VCM compared to the rat. Additionally, the rat is thought to have greater amounts of mEH compared to the mouse (Kitteringham et al., 1996; Krause et al., 1997). Thus, the rat likely has a greater ability to detoxify VCD to the non-toxic tetrol. Taken together, the rat has a lesser ability to bioactivate VCH and a greater ability to detoxify VCD, compared to the mouse, thus making the rat less susceptible to the ovotoxic effects of this chemical.

The role of ovarian metabolism in VCH-induced ovotoxicity is not known. Previous studies in our laboratory suggested that the mouse ovary does express mRNA and protein for various metabolic enzymes (Cyp 2E1, Cyp 2A, Cyp 2B, and mEH) thought to be involved in the hepatic metabolism of VCH. mRNA, total protein, and functional activity for these enzymes is altered following in vivo dosing with VCH or
VCD. Thus, the mouse ovary has the metabolic capacity to be involved in xenobiotic metabolism.

On the other hand, the role of ovarian metabolism in the rat is not known. Although studies by Flaws et al (1994b) showed the ability of pre-antral follicles from rats to detoxify VCD to the non-toxic tetrol metabolite, as measured by HPLC, the ability of the rat ovary to participate in bioactivation reactions of VCH is not known. Thus, the purposes of this study were to determine whether metabolic enzymes, specifically Cyp 2E1, Cyp 2A, and Cyp 2B, are functional in the rat ovary, and whether or not these enzyme activities are altered by in vivo dosing with either VCH, 1,2-VCM, or VCD.

EXPERIMENTAL METHODS:

Female Fischer 344 rats (d 28) were utilized for all studies. Animals were dosed daily (15 d) with either vehicle-control, VCH, 1,2-VCM, or VCD. Four h following the final dose, whole ovaries were removed. Total protein was isolated and the concentration determined. Specific activities for Cyp 2E1, Cyp 2A, and Cyp 2B were measured using model substrates. All methods are discussed in detail in Chapter 2.

RESULTS:

Cyp 2E1 Activity Assay: Specific activity for Cyp 2E1 was analyzed by hydroxylation of p-nitrophenol in whole ovarian homogenates from rats following in vivo dosing (15 daily doses) with vehicle-control, VCH, 1,2-VCM, or VCD. Cyp 2E1 activity in rat
ovaries could not be measured above baseline in any treatment group, despite the linearity of the reaction in hepatic microsomes obtained from mice.

Cyp 2A Activity Assay: No specific activity for Cyp 2A could be measured in ovarian homogenates obtained from rats in any treatment group. However, the assay was validated because the hydroxylation of coumarin to 7-hydroxycoumarin was linear in liver microsomes from mice.

Cyp 2B Activity Assay: Specific activity for Cyp 2B was evaluated by O-deethylation of 7-ethoxy-4-trifluoromethyl coumarin in whole ovarian homogenates obtained from rats dosed daily (15 d) with vehicle-control, VCH, 1.2-VCM, or VCD. Basal levels of Cyp 2B activity were above baseline (0.0001 to 0.3867 nmol/min/mg), however, the reaction was not linear in terms of incubation time or protein concentration. Specific activity for Cyp 2B was linear in hepatic microsomes obtained from mice.

DISCUSSION:

The reduced capacity of the rat liver to bioactivate VCH to 1.2-VCM, and subsequently VCD, is protective in terms of ovarian follicle loss. However, when rats are exposed to 1.2-VCM and/or VCD, follicle loss occurs. Although the liver is the primary organ involved in bioactivating 1.2-VCM to VCD, the relative contribution of the rat ovary in such reactions is not known. Our study was designed to evaluate the capacity of the rat ovary to participate in such reactions. Due to the lack of enzyme activity in the rat
ovary, our results suggest that it does not play a role in bioactivation of either VCH or 1.2-VCM. Specific activities for Cyp 2E1 and Cyp 2A were not detected above background in the rat ovary. Although, Cyp 2B activity was detected above baseline, enzyme kinetics were not linear, suggesting that this isoform is also not involved in VCH/1.2-VCM metabolism in the rat ovary.

The fact that the rat ovary does not appear to have the metabolic machinery necessary to bioactivate VCH/1.2-VCM is supported by Bengtsson et al. (1990). In those studies, the presence of various Cyp 450 isoforms was evaluated in ovarian microsomes obtained from rats following in vivo dosing with the ovotoxicant, DMBA. They concluded that the Cyp 450 isoforms, Cyp 2A, Cyp 2B, Cyp 1A1, Cyp 2D, and Cyp 3A, were not present in sufficient amounts to play a significant role in the ovarian metabolism of PAHs, as analyzed by Western blot analysis. The results presented here are further supported by in vitro studies performed in our laboratory, in which whole ovaries obtained from rats were incubated in vitro for 15 d with 1,2-VCM (20μM, 100 μM, 500 μM). Upon completion of the time course, the ovaries were fixed for histology, sectioned, and stained with hematoxylin and eosin for evaluation of the number of follicles by light microscopy. Analysis revealed that there were no statistically significant differences in the number of follicles (primordial, small primary, large primary) in 1,2-VCM-treated ovaries, compared to vehicle-control-treated ovaries. Taken together, these studies support the idea that the rat ovary does not have the metabolic capacity to bioactivate either VCH to 1.2-VCM or 1,2-VCM to VCD.
Although not evaluated in this study, the rat ovary likely plays a role in
detoxification reactions. In studies by Flaws *et al.* (1994b) the ability of follicles (F1 and F2) to metabolize $[^{14}C]$-VCD to the tetrol metabolite was evaluated. In immature rats, “conversion of VCD to tetrol by large pre-antral follicles (F2) was 1.5-fold greater than that by small pre-antral follicles (F1). There was no significant difference between the amount of VCD converted to tetrol by large pre-antral follicles compared with hepatocytes.” In adult rats, hepatocytes metabolized a significantly greater amount of $[^{14}C]$-VCD to the tetrol metabolite, compared to F1 and F2 follicles. Additionally, when the amounts of tetrol formed are directly compared between immature and mature rats, a significantly greater amount of tetrol was formed in all tissues (F1 and F2 follicles, hepatocytes) from mature rats, compared to those tissues from immature rats. Since rats are mature following 15 d of repeated dosing, the rat ovary is likely involved in detoxification reactions of VCD. However, because direct analysis of tetrol formation has not been studied in mouse follicles, we cannot directly compare the amounts of tetrol formed between species.

The ability of the mouse ovary to participate in bioactivation reactions is yet another factor contributing to the species specificity of the ovotoxic response. In terms of ovarian metabolism, the mouse ovary appears to be equipped with xenobiotic metabolizing enzymes, whereas the rat ovary does not. Lacking the necessary bioactivation enzymes may further explain why the rat is less susceptible to the ovotoxic effects of these chemicals, compared to the mouse. And, theoretically speaking, the rat ovary likely has more mEH compared to the mouse ovary (Kitteringham *et al.*, 1996;
Krause et al., 1997). This difference in detoxifying ability may further explain why the rat is less sensitive and the mouse is more sensitive to the ovotoxic effects of VCH and 1,2-VCM. This information collectively supports the greater susceptibility of mice, compared with rats, to these chemicals. Additionally, the lack of ovarian metabolism in the rat further emphasizes the role of hepatic metabolism in this species. At the same time, this strengthens the role of ovarian metabolism in VCH-induced ovotoxicity in the mouse.
Chapter 6

Summary: The Role of Ovarian Metabolism in 4-Vinylcyclohexene-Induced Ovotoxicity in B6C3F1 Mice

This dissertation work has contributed to the general understanding of ovarian metabolism, as well as its involvement in VCH-induced ovotoxicity. The data presented in the previous chapters support the hypothesis that the mouse ovary has the capacity (mRNA, total protein, and functional protein) to be involved in metabolic reactions (bioactivation or detoxification). The experimental approach allowed investigation of differential responses within targeted and non-targeted subpopulations of ovarian tissue. Interestingly, when the ovarian distribution of Cyp 2E1, Cyp 2A, Cyp 2B, and mEH was evaluated, a large amount of protein for all enzymes in the non-targeted interstitial cells was observed. This finding suggests an important physiologic and toxicologic function for this cell type that has not previously been characterized. Furthermore, since the interstitial cells are in the vascularized region of the ovary, it is likely that they would encounter toxicants traveling through the blood stream, including VCH, VCM, and VCD, and may affect their metabolism. Taken together, this ovarian cell type could either promote or protect against toxicity, depending on whether a more or less toxic metabolite is produced, respectively.
Our studies showed that repeated in vivo dosing with VCH or VCD altered the evaluated parameters: mRNA expression, total protein distribution, and functional activity (Table 6.1).

Table 6.1

<table>
<thead>
<tr>
<th>Metabolic Enzyme</th>
<th>mRNA Expression</th>
<th>Total Protein Distribution</th>
<th>Functional Activity</th>
</tr>
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<tbody>
<tr>
<td><strong>Cyp 2E1</strong></td>
<td>F1 $\rightarrow$ ↑VCH ↑VCD</td>
<td>Int cells $\rightarrow$ ↓VCH</td>
<td>Whole ovary $\rightarrow$ ↑VCH</td>
</tr>
<tr>
<td></td>
<td>F3 $\rightarrow$ ↑VCH</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyp 2A</strong></td>
<td>F1 $\rightarrow$ ↑VCH ↑VCD</td>
<td>Int cells $\rightarrow$ ↑VCH</td>
<td>Whole ovary $\rightarrow$ Not detected</td>
</tr>
<tr>
<td></td>
<td>Int cells $\rightarrow$ ↑VCH</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Cyp 2B</strong></td>
<td>F1 $\rightarrow$ ↑VCH</td>
<td>F1 (Small primary GC) $\rightarrow$ ↑VCH F3 (Antral GC) $\rightarrow$ ↑VCH</td>
<td>Whole ovary $\rightarrow$ No effect</td>
</tr>
<tr>
<td></td>
<td>F2 $\rightarrow$ ↑VCH ↑VCD</td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>F3 $\rightarrow$ ↑VCH↑VCD</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Int cells $\rightarrow$ ↓VCH</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>mEH</strong></td>
<td>F1 $\rightarrow$ ↑VCH ↑VCD</td>
<td>F2 (Theca cells) $\rightarrow$ ↑VCH ↑VCD</td>
<td>F1 $\rightarrow$ ↑VCH ↑VCD</td>
</tr>
<tr>
<td></td>
<td>F2 $\rightarrow$ ↑VCH↑VCD</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>F3 $\rightarrow$ ↑VCH↑VCD</td>
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Table 6.1: Summary of statistically significant changes in ovarian mRNA expression, total protein distribution, and functional activity for Cyp 2E1, Cyp 2A, Cyp 2B, and mEH following repeated dosing with VCH or VCD in mice. (p<0.05)
These findings support the role of ovarian metabolism in VCH-induced ovotoxicity. Again, of particular interest are the interstitial cells. Ovarian distribution of Cyp 2E1 and Cyp 2A were altered in the interstitial cells following VCH dosing, while expression of mEH was altered in the theca cells, which differentiate to form interstitial cells during atresia. Thus, the interstitial cells likely have an important function in both bioactivation and detoxification reactions. The data support the role of ovarian bioactivation, via Cyp 2E1 (Figure 6.1), and detoxification, via mEH (Figure 6.2), by the interstitial compartment. Although the ovary probably plays a greater role in detoxification reactions, compared to bioactivation reactions, further studies are required to investigate the involvement of these enzymes, and their relative contribution to the overall toxicity.

More experiments are required to further establish the roles of Cyp 2E1 and mEH in the ovarian bioactivation of VCH/VCM and detoxification of VCM/VCD, respectively. The use of transgenic mice would be ideal to answer such questions. Studies utilizing Cyp 2E1 deficient mice have helped researchers elucidate metabolic pathways for BD and acetaminophen. Cyp 2E1 deficient mice, exposed to either BD via inhalation or dosed with toxic amounts of acetaminophen, produced fewer toxic metabolites and less toxicity compared to wild type-controls (Jackson et al., 1999). mEH deficient mice have also been utilized in evaluating the effects of DMBA. These mice were unable to produce the toxic metabolite, were highly resistant to DMBA-mediated toxicity and carcinogenicity, and were completely protected from DMBA-induced
Figure 6.1 Proposed schematic for role of ovarian bioactivation of VCH or VCM. VCH or VCM is likely bioactivated in the ovary by Cyp 2E1 in the Int cells to the ovotoxic metabolite, VCD.
Figure 6.2 Proposed schematic for role of ovarian detoxification of VCM or VCD.

VCM or VCD is likely detoxified in the ovary by mEH in F1 follicles, F2 follicles, and Int cells to the non-toxic diol and tetrol metabolites, respectively.
tumorigenesis (Miyata et al., 1999). Thus, transgenic mice may also be useful in further
determining the contribution of ovarian bioactivation, via Cyp 2E1, and detoxification,
via mEH, of VCH and its epoxide metabolites. For example, transgenic mice could be
dosed in vivo with VCH/VCD via the standard dosing regimen (15 d), their ovaries
removed, and functional activity determined for Cyp 2E1, Cyp 2A, Cyp 2B, and mEH.
The results would provide additional support for the involvement of various Cyp 450
isoforms, as well as a general comparison of bioactivation and detoxification capabilities.
Alternately, ovaries from untreated transgenic mice could be utilized for whole ovarian
culture studies. Follicle loss could be evaluated following in vitro incubation with VCM,
which may strengthen the relative contribution of bioactivation and detoxification in the
ovary. Due to the limited accessibility and the high cost of transgenic mice, the role of
Cyp 2E1 and mEH in VCM-induced follicle loss could be assessed via the in vitro culture
approach in B6C3F1 mice. The broad-spectrum Cyp 450 inhibitor, 1-
aminobenzotriazole, the specific Cyp 2E1 inhibitor, 1,2-trans-dichloroethylene, as well
as the mEH inhibitor, CHE, could be added to the culture medium and the effects
evaluated. Because there is a delicate balance between bioactivation and detoxification
that dictates protection versus toxicity, it would also be important to perform dosing
studies of varying durations. These studies may further explain the involvement of other
Cyp 450 isoforms, as induction may be seen for these enzymes at times other than 15 d.

Ultimately, to study the relative contribution of ovarian versus hepatic
metabolism, the direct ovarian metabolism of VCH, VCM, and VCD needs to be
evaluated, and formation of metabolites identified and quantified. Even though the
analytical techniques would prove challenging, verification of metabolites is the only way to establish ovarian metabolism of VCH and/or VCM. Once established in whole ovarian homogenates, the involvement of specific sizes of ovarian follicles, as well as interstitial cells, in the ovarian metabolism of these substrates could be determined. These parameters could also be evaluated in Cyp 2E1 and mEH deficient mice.

Although the previously described experiments would verify the involvement of ovarian metabolism in VCH-induced ovotoxicity, this information needs to be applied more globally. Ideally, other ovotoxic agents, such as BD epoxides would be evaluated. Since humans are widely exposed to BD, this would make the findings more relevant to human exposure.

As previously described, mice and rats are inherently different in their ovarian metabolic capabilities. There is evidence that Cyp 2E1 activity is induced in ovaries from VCH-treated mice, whereas Cyp 2E1 activity is not detected in rat ovaries. This further suggests that mice are more susceptible to the ovotoxic effects of VCH/VCD, compared to rats. Since in many toxicology studies, researchers must extrapolate results obtained from rodent studies to predict what may happen in humans, it would be beneficial to add human information to this species comparison. Two investigators have detected mEH in the human ovary. Although it is anticipated that Cyp 450 enzymes are also present in the human ovary, specific isoforms, as well as the ovarian distribution of these enzymes, is not known. Characterization of these enzyme profiles in humans would prove useful. mRNA expression, total protein, and functional activity could be determined in different cell types of the human ovary, since samples can be obtained
from *in vitro* fertilization clinics. Furthermore, *in vitro* metabolism of VCH, as well as other compounds, could be monitored by metabolite formation and identification. This sort of human information would prove invaluable, as relative risks to women exposed to these chemicals in the work place could be determined.
APPENDIX A

USE OF AN IN VITRO CULTURE SYSTEM IN WHOLE OVARIES TO EVALUATE THE ROLE OF OVARIAN METABOLISM OF 4-VINYLCYCLOHEXENE EPOXIDES IN MICE

INTRODUCTION:

The use of whole organ culture is a powerful technique because many of the intricate, yet complicated processes that occur in vivo are eliminated from the system. Although studies are often carried out in greatly simplified conditions in comparison to the in vivo approach, many fundamental questions can be addressed, as long as there is "continued development or orderly persistence of differentiated structure in the explanted organ which is thus able to exercise in vitro some of its in vivo function" (Fainstat, 1968). The use of whole organ culture has been successfully carried out in various tissues, including the gonads. These studies have been particularly useful in understanding the complex interactions in reproductive biology. For instance, deciphering the many external and internal factors that contribute to follicular growth has made it difficult to study follicular dynamics. To make the confounding variables easier to interpret, investigators developed methods to cultivate whole ovaries to study the growth and differentiation of germ cells.

The earliest studies using whole ovarian cultures were done by Martinovitch in 1938. He utilized an in vitro approach to study follicle development in whole ovaries
from rats and mice, ranging in age from GD 16 to PND 4. Ovaries were cultured in watch glasses with medium containing chicken embryo extracts and chicken plasma. Oocytes did grow and differentiate, however, development of the entire follicle was suppressed. Additionally, the more highly developed the ovary at the initiation of in vitro culture, the more it differentiated. Martinovitch concluded that explanted ovaries can survive in culture for approximately 4 weeks before becoming necrotic. Importantly, the success of these cultures established reason to continue the pursuit to improve such techniques.

Although ovaries did survive in vitro, the complexity of the medium still made it difficult to distinguish which factors were necessary for follicle development. In 1968, Fainstat was the first to culture post-natal rat ovaries in chemically defined medium, free of "unknown" factors in natural, serum-containing medium. In these studies, ovaries from rats 2 to 25 d old were utilized. Post-natal rat ovaries survived in culture up to 14 d, when optimal amounts of oxygen, carbon dioxide, and nitrogen were used. Follicle oocytes grew and surrounding granulosa cells proliferated. Interestingly, ovaries obtained from younger animals had a better outcome in culture, compared to ovaries from older animals.

More recently, studies by Wandji et al (1996, 1997) have shown follicle growth from isolated primordial follicles obtained from cows and baboons in vitro. More than 80% of primordial follicles progressed to the primary follicle stage within 2 days of culture in both species. After 7 days of culture in both species, there was further increase in follicle diameter, an increase in the number of granulosa cells, as well as the presence
of proliferating cell nuclear antigen (PCNA), thought to be a reliable marker of cell proliferation (Liu et al., 1989; Hall et al., 1990). These observations support in vitro follicle development, however, there are likely factors from other ovarian compartments that contribute to this unique process, and are not accounted for in cultures of isolated follicle populations.

Many of the initial whole ovarian culture studies evaluated follicle development as a means to better understand ovarian physiology. However, this organ culture system seemed ideal to also ask biochemical questions. Baker and Neal (1969) were pioneers in utilizing this technique to examine toxicological endpoints. Ovaries from mice, rats, monkeys, and humans were irradiated in order to compare the sensitivity of the gonads to radiation. Their results showed that irradiation effectively eliminated the primordial follicle pool in cultured ovaries from rats (150 or 300 Rads. over 7 d), monkeys (5000 Rads. over 7 d), and humans (4000 Rads. over 7 d). Mouse ovaries, which were difficult to culture, were essentially resistant to irradiation treatment (100 Rads. over 7 d). Additionally, the doses used correlated well with in vivo dosing studies, making the in vitro model a good indicator of in vivo response.

Only recently have researchers re-discovered the value of whole ovarian culture. Parrott et al (1999) and Nilsson et al (2001) refined the organ culture set-up, in which ovaries from PND 4 rats were placed on membranes and floated on top of culture medium. Their studies evaluated the effects of several exogenous factors, such as insulin, growth factors, and stem cell factors on follicle development. Following in vitro culture
for 14 d, the ovaries were fixed in Bouin’s fixative, sectioned, stained with hematoxylin and eosin, and the number of follicles in each developmental stage counted.

Previous studies in our laboratory have further exploited the ovary culture system, developed by Dr. Skinner’s group, to study the effects of the ovarian toxicant, VCD (Devine et al. 2002). Since VCD selectively targets the primordial and small primary follicles, this model system was advantageous, since ovaries obtained from 4 d old rats are virtually 100% primordial follicles. Ovaries were treated in vitro with various concentrations of VCD (1-100 μM) for 2 to 15 days. VCD decreased the number of small ovarian follicles in a dose and time-dependent manner. These results demonstrated that the in vitro culture system of whole ovaries is useful for studying the direct effects of VCD, and may be useful to study other reproductive toxicants.

Since the in vitro ovary culture system eliminates the involvement of other organs, such as the liver, this set-up was an optimal tool to address the role of ovarian metabolism in VCH-induced follicle loss. Although the culture system has not been previously characterized in mice, our studies were designed to evaluate the in vitro effects of 1,2-VCM and VCD in mouse ovaries. The purpose of our studies was to 1) evaluate the general morphology of mouse ovaries following culture for 15 d in control medium, 2) evaluate the in vitro effects of 1,2-VCM and VCD on follicle numbers, and 3) determine if metabolic enzymes (Cyp 2E1, Cyp 2A, and Cyp 2B) are present in ovaries from PND 4 mice.
EXPERIMENTAL METHODS:

Female B6C3F1 mice (PND 4) were utilized for all studies. Pups were killed via euthanization and ovaries removed. Whole ovaries (1 or 2) were floated on membranes atop of media containing the appropriate treatment in 4-well culture plates. A small amount of medium was placed on top of the ovary to prevent the tissue from drying out and to optimize exchange of nutrients and oxygen. Plates were incubated in a humidified environment (95% air, 5% CO₂) for 15 days. Medium and treatments (vehicle control, 30 μM VCD, 250 μM 1.2-VCM, 750 μM 1.2-VCM, or 1000 μM 1.2-VCM) were changed every other day. Due to the volatility of VCH, 1.2-VCM was used to evaluate the role of ovarian metabolism, particularly bioactivation, in this system. Following 15 days of in vitro incubation, the ovaries were fixed in Bouin’s fixative for histology. The ovaries were sectioned, stained, and the number of follicles (primordial, small primary, large primary) counted.

Additionally, to verify the presence of different metabolic enzymes in ovaries from PND 4 mice, ovaries were fixed for immunohistochemistry. Utilizing confocal microscopy, the ovarian distribution of Cyp 2E1, Cyp 2A, Cyp 2B, and mEH was evaluated. All methods are described in detail in Chapter 2.

RESULTS:

General Morphology: Following 15 d of in vitro incubation, the overall appearance of the ovaries was evaluated, as a general measure of viability. The ovaries appeared to be healthy and there was little evidence of necrosis. Follicles were well developed,
containing oocytes surrounded by granulosa cells (fusiform or cuboidal). Larger secondary and antral follicles were not present, as expected, since gonadotropins were not added to the culture medium.

Ovarian Follicle Counts: The number of primordial, small primary, and large primary follicles were counted (every 6th section) in cultured ovaries following 15 d of *in vitro* incubation in medium containing either Con. 1,2-VCM, or VCD. The summary of the preliminary results are presented in Table A.1, as well as in Figures A.1, A.2, and A.3.

Table A.1

<table>
<thead>
<tr>
<th><em>In Vitro</em> Treatment Group</th>
<th># of Primordial Follicles</th>
<th># of Small Primary Follicles</th>
<th># of Large Primary Follicles</th>
</tr>
</thead>
<tbody>
<tr>
<td>Con</td>
<td>462</td>
<td>89</td>
<td>11</td>
</tr>
<tr>
<td>250 μM 1,2-VCM</td>
<td>181</td>
<td>29.5</td>
<td>9.5</td>
</tr>
<tr>
<td>750 μM 1,2-VCM</td>
<td>66.5</td>
<td>31.5</td>
<td>8</td>
</tr>
<tr>
<td>1000 μM 1,2-VCM</td>
<td>52.7</td>
<td>18</td>
<td>7.7</td>
</tr>
<tr>
<td>30 μM VCD</td>
<td>2</td>
<td>1.4</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table A.1: Summary of preliminary ovarian follicle counts in mice.
Figure A.1: Primordial follicle counts from cultured ovaries. Ovaries from PND 4 mice were collected and cultured *in vitro* for 15 d in the presence of untreated media (Con) or media containing 1.2-VCM (250, 750, or 1000 μM), or VCD (30 μM). Ovarian follicles were classified and counted in every 6th section, as described in Chapter 2. Due to limited sample size, statistics were not performed.
Figure A.2: Small primary follicle counts from cultured ovaries. Ovaries from PND 4 mice were collected and cultured in vitro for 15 d in the presence of untreated media (Con) or media containing 1,2-VCM (250, 750, or 1000 μM), or VCD (30 μM). Ovarian follicles were classified and counted in every 6th section, as described in Chapter 2. Due to limited sample size, statistics were not performed.
Figure A.3: Large primary follicle counts from cultured ovaries. Ovaries from PND 4 mice were collected and cultured *in vitro* for 15 d in the presence of untreated media (Con) or media containing 1,2-VCM (250, 750, or 1000 μM), or VCD (30 μM). Ovarian follicles were classified and counted in every 6th section, as described in Chapter 2. Due to limited sample size, statistics were not performed.
Figure A.4: **Micrographs from cultured ovaries.** Ovaries from PND 4 mice were collected and cultured *in vitro* for 15 d in the presence of untreated media (A) or media containing 1000 μM 1.2-VCM (B). Following incubation, ovaries were placed in Bouin’s fixative. Ovarian follicles were classified and counted in every 6th section, as described in Chapter 2. All images were captured at 10X magnification.
Figure A.4
Confocal Microscopy: Total protein for Cyp 2E1, Cyp 2A, Cyp 2B, and mEH was visualized in the PND 4 mouse ovary. Protein was present in oocytes, granulosa cells, and the surrounding Int cells. Compared to older animals, however, the staining intensity was not as strong. (Figure A.5). No autofluorescence was seen in unstained, coverslipped ovarian sections at \( \lambda = 647 \) nm.
Figure A.5: **Ovarian distribution of metabolic enzymes in ovaries from post-natal day 4 old mice.** Ovaries were removed from untreated B6C3F₁ mice (PND 4). Tissue was fixed in 4% formalin, sectioned (5 μm thick, every 7th section), and incubated with primary antibodies for Cyp 2E1, Cyp 2A, Cyp 2B, or mEH. Immunofluorescence was visualized on a Leica confocal microscope with a xenon light source and the intensity was determined via an argon-krypton laser projected through the tissue into a photomultiplier tube at λ = 488 nm for YOYO-1 (green; DNA stain) and λ = 647 nm for Cy-5 (red: Cyp 2E1, Cyp 2A, Cyp 2B, or mEH). All images were captured at 20X magnification. A) Cyp 2E1. B) Cyp 2A. C) Cyp 2B. and D) mEH
Figure A.5

Cyp 2E1

Cyp 2A

Cyp 2B

mEH
DISCUSSION:

Use of an in vitro culture system is an ideal approach to evaluate the direct effects of ovarian toxicants. Similar to the results in the rat, our studies suggested that VCD does target and destroy the same population of follicles (primordial and small primary) that are depleted by repeated in vivo dosing with VCD. Following 15 d of incubation, VCD effectively reduced the number of primordial, small primary, and large primary follicles, compared to control.

Our results are encouraging in that in vitro incubation with 1,2-VCM did cause follicle loss, relative to control, and follicle loss was dose-dependent. This suggests that in vivo, bioactivation of 1,2-VCM to VCD can occur in the ovary, in addition to the liver. However, the relative contribution of ovarian versus hepatic metabolism is not known, nor is the relative contribution of ovarian detoxification. Previous studies by Flaws et al. (1994b) showed the ability of the rat ovary to metabolize VCD to the inactive tetrol metabolite, while we previously suggested the role of detoxification in the mouse ovary, as evidenced by an induction in mEH activity in the targeted population of follicles following VCH and VCD dosing, in vivo. Additionally, when one examines the basal activities of mEH in distinct ovarian compartments (F1, 34.2 ± 15.5 nmol/min/mg; F2, 147.7 ± 42.1 nmol/min/mg; F3, 19.3 ± 9 nmol/min/mg; Int, 16.3 ± 6.8 nmol/min/mg of protein) compared to Cyp 450 enzyme activity in whole ovaries (Cyp 2E1, 0.073 ± 0.02 nmol/min/mg; Cyp 2B, 0.004 ± 0.001 nmol/min/mg or protein) from mice, it is obvious that the detoxification abilities of the mouse ovary are orders of magnitude greater than the bioactivation abilities. To further investigate the role of ovarian detoxification in the
*in vitro* culture system, use of a mEH inhibitor, cyclohexene oxide, would prove beneficial.

While performing this set of experiments, we encountered several problems that complicated interpretation of the results. Occasionally, ovaries became bacterially contaminated and could not be used for histological analysis. There were additional problems with histology. Because of the very small size of the ovary, several samples were simply lost during handling by the histology technical staff. On occasion, the histology personnel were aware of the problem. However, this was not consistent, because we received several slides that were labeled, yet there were no ovarian sections on the slide. Also, several ovarian sections were torn and regions of the ovary were folded on top of each other, making accurate analysis of the number of follicles impossible. The initial orientation of the ovary is important in the resulting number of sections obtained. Ideally, the sections should be cut horizontally, with the ovary staying "flat", thus giving the largest cross-section of the ovary as possible. However, if the ovary is cut vertically, the cross-section is much smaller (Figure A.6). Theoretically, there should have been a greater number of sections obtained when the ovary was cut in this manner. Unfortunately, there were not. Thus, it is possible that sections, or ribbons, of tissue were lost. When one is evaluating the absolute number of follicles in a given section/ovary, all of these parameters suddenly have great impact on the final results. In our case, although we see trends for an effect of 1,2-VCM on follicle number, these values cannot be considered reliable without additional studies.
Figure A.6: Orientation of ovary during histological sectioning. A horizontally oriented ovary is optimal, resulting in sections with the largest cross-sectional area. Vertically oriented ovaries result in a greater number of sections. This is misleading when every 6th section is evaluated for the absolute number of follicles contained in a particular section.
When evaluating these studies, one also has to consider the age of the animal in the experiment. In the *in vivo* studies, the animals were 28 – 42 days of age. However, in the *in vitro* culture system, ovaries from PND 4 animals were utilized. And, it is a well reported phenomenon in the literature that Cyp 450 profiles change shortly after birth (Muhktar *et al.*, 1978). Thus, we were not sure if the PND 4 ovary expressed the same enzymes as older animals. Our confocal microscopy analysis revealed that Cyp 2E1, Cyp 2A, Cyp 2B, and mEH are present in the PND 4 mouse ovary. However, the expression profile was different compared to older mice. For instance, there was less staining intensity in the Int cells, a region of the ovary where there was intense staining in ovaries from older animals. Additionally, even though these metabolic enzymes were present in the PND 4 mouse ovary, it is not known whether or not these enzymes were catalytically functional.

We are planning on repeating this set of experiments. In the study design, we will utilize additional doses of 1,2-VCM and VCD in an attempt to see a better dose response. Additionally, we will use the mEH inhibitor, CHE. The treatment groups will be as follows: Control, 15 μM VCD, 30 μM VCD, 2 mM CHE, 125 μM 1,2-VCM ± 2 mM CHE, 250 μM 1,2-VCM ± 2 mM CHE, 500 μM 1,2-VCM ± 2 mM CHE, 750 μM 1,2-VCM ± 2 mM CHE, or 1000 μM 1,2-VCM ± 2 mM CHE. We are hopeful that we will not encounter any of our previous problems, and that we will be better able to elucidate the role of ovarian metabolism in VCH/VCM/VCD-induced ovotoxicity.
REFERENCES


