INVOLVEMENT OF THE ESTROGEN RECEPTOR AND ARYL HYDROCARBON RECEPTOR IN 4-VINYLCYCLOHEXENE DIEPOXIDE-INDUCED OVOTOXICITY IN F344 RATS

By

Kary Ellen Thompson

A Dissertation Submitted to the Faculty of

GRADUATE INTERDISCIPLINARY PROGRAM IN PHYSIOLOGICAL SCIENCES

In Partial Fulfillment of the Requirements for the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2001

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Kary Ellen Thompson

entitled Involvement of the estrogen receptor and aryl hydrocarbon receptor in 4-vinylcyclohexene diepoxide-induced ovotoxicity in F344 rats.

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Patricia B. Hoyer, Ph.D.	Date 11/19/01
Adele M. Turzillo, Ph.D.	Date 11/19/01
Ping Xia, M.D., Ph.D.	Date 11/19/01
I. Glenn Sipes, Ph.D.	Date 19 Nov 01
Qin Chen, Ph.D.	Date 11-19-01

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director Patricia B. Hoyer, Ph.D. Date 11/19/01

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advanced degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Kay C. Thompson

ACKNOWLEDGEMENTS

First and foremost, I wish to thank my mentor, Dr. Patricia Hoyer. Not only did she take me under her wing when I arrived here in Tucson wide-eyed and naive, but helped me grow as a person and a scientist. Pat is a model advisor, making sure that not only was my research on track, but also, making my career development skills important as well. Pat is a wonderful and consistent source of feedback and support, from trouble shooting an experiment to advising on postdoc positions. I feel so lucky to have completed my dissertation in her lab. Next, I would like to acknowledge Dr. Adele Turzillo for her support. She is always available for questions about everything. Her help with my research was invaluable. I would also like to thank the rest of my committee for their time and input: Drs. Ping Xia, Glenn Sipes, and Qin Chen.

This work was not done alone. I am indebted to the members of Pat's lab, "The Hoyer Warriors." Sherri Borman, while she is now in Oregon, was the person who so open heartedly welcomed me into this lab. She is wonderful friend and roommate. I miss her everyday. Next, Ellen Cannady, not only for showing up in the lab at the perfect time, but for being an amazing source of support in all aspects of my life. I can rely on Ellen for scientific guidance, a sympathetic ear, a caring friendship, an evening of scrapbooking or knitting, a light-hearted conversation about baskets, or an evening of stress relieving racquetball. I can't forget to acknowledge my other Longaberger buddy, Debbie Pierce. I'm so glad that we're still friends even after the car trip to Taos, New Mexico!! I must include Patty Christian as another important contributor to my success. Patty is indispensable as a constant helping hand. She is truly a thoughtful person and I am not sure how she manages to take such good care of everyone in Pat's lab.

Graduate school has been an amazing experience for me. During this time, I actually grew up and became the person I am today. And an important part of that development came from outside of the lab. I was very lucky to be part of a wonderful graduate program filled with incredible people. Holly Lopez, our program coordinator, was so patient; her door was always open. Next, my roommate, Kameha Kidd, was another significant person. Kameha not only overlooked more than one mess I left in the apartment, but has been a very understanding, caring, and supporting friend. Finally, in graduate school, I met the two most phenomenal friends I could ever hope to have in a lifetime. Gary & Vicki Sutherland are the two most dedicated and caring people I have ever known. I value their friendship immensely. I can count on them for anything and I mean ANYTHING. There are few people you come across in this world that have such an impact on you...Gary and Vicki were there from the first day I got into town, and I am going to make sure that even when I am across the country, that I will still keep them close to my heart.

Finally and most importantly, I must thank my family. My parents never questioned my motivation to continue school after college. In fact, they have always supported every decision I have made and tried their best to make things as easy and smooth as possible for me. Knowing that I could come home at anytime or that they would come out here made moving and being across the US from them so much better. I hope I have made you proud Mom and Dad; I love you!!

This is for Mom and Dad,

Richard and Sharon Thompson

TABLE OF CONTENTS

TABLE OF CONTENTS - *continued*

LIST OF FIGURES

LIST OF FIGURES - *continued*

LIST OF TABLES

ABSTRACT

Women are born with a finite number of primordial follicles, the smallest follicles in the ovary. Once these follicles are destroyed, they cannot be replaced and after extensive loss, ovarian failure (menopause) can occur. The industrial chemical 4-vinylcyclohexene diepoxide (VCD) induces depletion of these follicles and causes premature ovarian failure in rats. VCD-induced ovotoxicity has been found to accelerate a natural process in the ovary, atresia, which occurs via apoptosis. The mechanism(s) by which VCD enhances follicular atresia are unknown; however, it has been shown to alter the expression of several genes and proteins associated with apoptosis. While downstream signaling events of VCD are becoming identified, the early signaling events of this pathway have not yet been determined, but may involve a receptor-mediated cascade. Therefore, these studies tested the hypothesis that VCD-induced ovotoxicity involves a nuclear receptor-mediated pathway that leads to increased atresia. Concurrent treatment of rats with VCD and estradiol selectively protected primary follicles from loss by an estrogen receptor-mediated mechanism via a reduction of caspase-3-induced apoptosis. VCD does not alter ER number, affinity, circulating estradiol levels, or directly bind ERβ. Concurrent dosing of rats with VCD and an AhR antagonist prevented primordial and primary follicle loss via a reduction in caspase-3-induced apoptosis. Repeated dosing with VCD was shown to up-regulate expression of *AhR* mRNA; however, VCD did not alter expression of AhR-mediated genes *glutathione-S-transferase Ya1* or *Ya2* nor CYP 1A1 protein. AhR-deficient mice were still susceptible to VCD-induced follicle loss. Repeated dosing with VCD reduced Heat Shock Protein

(HSP) 90 expression in small primary follicles. Analogs of the ER and AhR did not alter HSP90 protein, nor did a loss of HSP90 function induce follicle loss or potentiate VCD-induced follicle depletion. While the ER, AhR, and HSP90 are all co-localized in the oocyte nucleus of primordial and primary follicles, no evidence was seen to support that these proteins are interacting. Taken together, the ER is able to prevent VCD-induced ovotoxicity in primary follicles, the AhR is not required for VCD-induced follicle loss, and HSP90 does not appear to play a central role in follicle depletion caused by VCD.

CHAPTER 1

INTRODUCTION

OVARIAN FOLLICULAR DEVELOPMENT

Successful reproduction in the female requires synchronous function of many organs throughout the body including the hypothalamus, pituitary gland, uterus, cervix, and ovary. Critical to this process is the ovary, for it contains the germ cells as well as produces most of the sex steroid hormones required to support female reproductive function. The basic unit of the ovary is the follicle, which consists of an oocyte surrounded by granulosa cells. Women are born with a finite number of primordial follicles, the earliest stage of follicular development. During reproductive life, immature primordial follicles are recruited from this pool following an unknown signal and begin to develop into primary follicles. Granulosa cells proliferate in number and form multiple layers of cells around the growing oocyte. In the final stages of development, an antrum forms in the granulosa cell compartment and production of the steroids 17β-estradiol is peak, eventually forming a preovulatory follicle ready for ovulation.

Formation of the follicular pool

Early in mammalian fetal development, primordial germ cells migrate into the undifferentiated gonad from the urogenital ridge and undergo rapid hyperplasia and mitosis (Byskov, 1986; Hirshfield, 1991a; Monniaux *et al.*, 1997). Somatic cells that comprise the follicle are formed in the embryonic indifferent gonad during this time as well. Once rested in the genital ridge, the germ cells, now termed oogonia, and the somatic compartment begin to intermingle within the tissue. Unlike males, mitosis of germ cells in females only occurs during embryonic development; therefore women are born with a finite number of oocytes.

Following an unknown signal at the end of the first trimester of human fetal development, oogonia stop dividing by mitosis and enter meiosis (Picton *et al.*, 1998). However, the meiotic process is not completed at that time. Rather, the oogonia progress through the meiotic prophase stages of leptotene, zygotene, and pachytene, and then are arrested in the diplotene phase of meiosis I (Hirshfield, 1991a). Oocytes remain arrested in meiotic prophase I until ovulation or degeneration occurs (Richards, 1980). Accompanying the initiation of oocyte meiosis is a mass attrition of germ cells. This germ cell wastage, from 6 months gestational age to birth, causes the number of oocytes to be reduced from about 7 million to approximately 1 million (Baker, 1963).

Near or just after birth in humans, each oocyte becomes surrounded by a single layer of flattened pre-granulosa cells and a basement membrane (Hirshfield, 1991a; Gondos, 1970; Pederson and Peters, 1968). This collection of cells is termed the

primordial follicle and forms the pool of follicles awaiting further development in the mature postnatal ovary. The basement membrane forms around the primordial follicle and separates the follicle from the interstitial, vascular, and stromal portions of the ovary (Hirshfield, 1991a; Guraya, 1985). Primordial follicles are not steroidogenic and are considered quiescent; however, they are still metabolically active, as marked by RNA and protein synthesis (Guraya, 1985; Picton *et al.*, 1998).

Primary follicle development

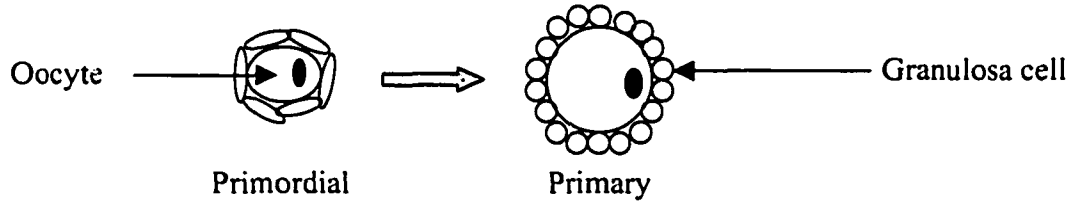


Figure 1.1. Primordial to primary follicular changes. (Fraction 1 follicles)

The signal for the initiation of primordial follicle development is unknown; however, it does not appear to come from a neural or adrenal source since mammalian follicles will develop *in vitro* in the absence of neurofactors or adrenal steroids (Eshkol and Lunenfeld, 1972; Lunenfeld and Eshkol, 1969). Following this unknown signal about 3 months prior to ovulation, a cohort of follicles (about 300 in women) begin to develop, however this cohort represents only a small portion of the total ovarian follicular reserve (Hirshfield, 1991a; Lunenfeld and Insler, 1993). In humans, follicles can spend

up to 50 years in the primordial stage before they are recruited for development or undergo attrition (Hirshfield, 1991a). Upon activation for development, animal studies have shown the first detectable changes in the primordial follicle involve a substantial increase in RNA and protein synthesis which causes the oocyte to grow in diameter, from 15μ to 100μ (Erickson, 1978). In mammals, at this point, the oocyte itself is fully differentiated and capable of meiotic completion (Erickson, 1978). This marked precocious oocyte maturation is in contrast to the rest of the somatic portions of the follicle which complete most of their development at a later stage. Early oocyte maturation is a hallmark of mammalian folliculogenesis. Following its growth, the mammalian oocyte becomes surrounded by a zona pellucida, a thick, acellular, glycoprotein matrix (Guraya, 1985). The squamous, flattened granulosa cells surrounding the oocyte become cuboidal in shape and mitotically active.

Secondary and antral follicle development

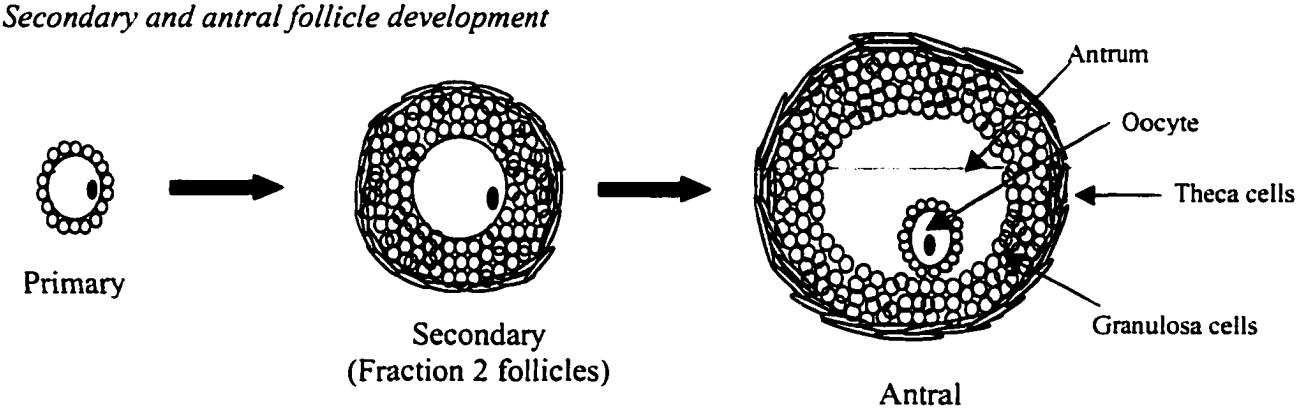


Figure 1.2. Primary follicular development to later stage follicles.

As granulosa cells continue to proliferate in primary follicles, they form multiple layers of cells around the oocyte. The presence of two or more layers of granulosa cells designates the follicle as a secondary follicle. In rats, the expression of follicle stimulating hormone (FSH), estrogen, and testosterone hormone receptors, as well as gap junctions, occurs during this phase of secondary follicle development (Erickson, 1978).

During this stage in mammalian follicugenesis, theca interna cells become associated with the follicle as concentric rings on the outside of the basement membrane. The theca interna cells contain a capillary network and are steroidogenic (Erickson, 1978). The acquired exposure of the follicle to blood plasma components causes a variety of effects. The follicle has expressed receptors for FSH, estradiol, and testosterone. Additionally, the vascular theca compartment is now exposed to these hormones. As a result, subsequent development of the follicle is gonadotropin dependent. At the same stage, theca interna cells begin to produce progestins and androgens (Basset, 1943; Hirshfield, 1991a,b).

Most of the follicular growth during this time is within the granulosa cell compartment. Continual addition of granulosa cells causes the follicle to grow from 125 μm to over 250 μm with little change in the oocyte size. The theca and granulosa cells together produce hormones which promote and support the differentiation of the secondary follicle to an antral follicle (Erickson, 1978). Hormones induce several changes in the follicle as this transformation occurs. First, FSH causes the formation of a fluid-filled antrum within the granulosa cell compartment. In humans, this cavity can increase the size of the follicle up to 2 cm in diameter (Erickson, 1978). Follicular fluid

contains mucopolysaccharides secreted by granulosa cells as well as specific proteins and hormones accumulated from plasma (Erickson, 1978). Second, FSH induces the enzyme aromatase, an important catalyst in estradiol synthesis. Estradiol is a critical hormone in ovarian and neuroendocrine function. Third, FSH induces the expression of luteinizing hormone (LH) receptors. LH receptors reach a maximum in the preovulatory follicle (Erickson, 1978).

Only some of the follicles recruited ever reach the final stages of follicular maturation. Through unknown controls and signals, usually only one follicle, termed the dominant follicle, is selected and eventually ovulated in women. Following the LH surge, the oocyte resumes genetic development and finishes the first meiotic division and begins meiosis II. However, the oocyte again does not finish the meiotic process as it is then arrested in metaphase of meiosis II. The second meiotic division will not be completed until fertilization.

ATRESIA

Normally only one follicle is ovulated each month in humans. The rest of the recruited follicles undergo a degenerative process termed atresia. Over 99% of follicles initially formed in the ovary die due to atresia, and on average, only 400-600 oocytes will ever be ovulated over the course of a woman's life. Germ cells can undergo atresia as primordial germ cells during embryonic life, as oogonia in the developing ovary, and at all the different phases of follicular development in the mature postnatal ovary (Hseuh *et al.*, 1994; Kaipia and Hseuh, 1997). In humans, germ cells number about seven million

before attrition begins at 6 months gestational age. At birth, this number has become reduced to 1-2 million oocytes, and then by puberty, to two-four hundred thousand oocytes (Block, 1952; Baker, 1963; Kaipia and Hsueh, 1997).

Based on morphological and biochemical studies, it has been determined that atresia occurs through the physiological cell death pathway, apoptosis (Hughes and Gorospe, 1991). This controlled cell death pathway is in contrast to necrosis, which occurs in response to injury and trauma and results in localized inflammation as cellular contents are released into the intracellular space (Hseuh *et al.*, 1994). In necrotic cell death, ATP generation, gene expression, and protein regulation does not occur. However, in apoptosis, programmed cell death occurs during normal embryo development to sculpt the body shape and in adult tissues to control cell turnover (Kaipia and Hsueh, 1997). Apoptosis is a regulated, energy dependent method for the body to eliminate cells in a non-traumatic way and is distinguished by characteristic changes in the morphology of the nucleus and cytoplasm (Kaipia and Hsueh, 1997). Based on immunohistochemistry, typical patterns of apoptosis include chromatin condensation, nuclear membrane disruption, plasma membrane shrinkage and blebbing, and the appearance of pyknotic nuclei and apoptotic bodies (Wyllie, 1987; Kaipia and Hsueh, 1997). A hallmark molecular clue for identification of apoptosis is endonuclease cleavage of DNA into fragments containing multiples of 185-200 basepairs (Wyllie *et al.*, 1980). This produces a characteristic laddered pattern of genomic DNA migration upon electrophoretic separation.

Ovarian follicular degeneration occurs in both somatic and germ cells of the follicle by a hormonally controlled and coordinated process (Kaipia and Hseuh, 1997). The specific control between follicular survival and atresia by apoptosis is not well understood. Many studies have examined the mechanism in large antral follicles. The primary site of apoptosis in follicular atresia is thought to be the granulosa cells (Billig *et al.*, 1993). Interleukin-1β (IL-1β), nitric oxide (NO), estrogens, activin, epidermal growth factor (EGF), gonadotropins (FSH, LH, hCG), and insulin-like growth factor-I (IGF-1) are all reported to suppress apoptotic DNA fragmentation in preovulatory follicles while androgens have the opposite effect (Tilly *et al.*, 1992; Chun *et al.*, 1994; Chun *et al.*, 1995; Chun and Hseuh, 1998). Additionally, tumor necrosis factor-α, interleukin-6, and gonadotropin releasing hormone (GnRH) promote apoptosis in large follicles. The factors which regulate follicular (granulosa cell) survival appear to differ for different stage follicles. In preantral follicles, through an unknown pathway, cGMP is able to prevent follicular apoptosis *in vitro*. In early antral follicles, FSH is the primary protective signal and mimicked by cAMP analogs. Preovulatory follicles have redundant pathways to ensure protection from atresia, and are protected by most all of the hormones and cytokine factors listed above (FSH, LH/hCG, GH, IGF, EGF, basic fibroblast growth factor, IL-1β, NO; Kaipia and Hseuh, 1997).

MENOPAUSE

When all follicles have been lost due to ovulation or atresia, ovarian failure (menopause in women) occurs. Menses cease and the reproductive lifespan of the woman has ended (Gosden, 1987). The mean age of menopause is 50-51 years (Gray, 1976). As a result of loss of follicles, ovarian steroid production ceases, and thereby reduces the negative feedback to the hypothalamus and pituitary. Subsequently, there is a rise in both circulating LH and FSH levels (Gosden, 1987). Menopause has been associated with an increased risk for a variety of health conditions including cardiovascular disease, stroke, and osteoporosis. A number of factors play a role in the age at the onset of menopause. Precocious follicle loss can be caused by genetic predisposition, **cytotoxic substances**, ionizing radiation, various disease states, and auto-antibodies (Gosden, 1987).

Menopause is associated with an increased risk for many health conditions. The incidence and prevalence of cardiovascular disease/death and atherosclerosis is higher after menopause (Joakimsen *et al.*, 2000). Thus, women that undergo **early** menopause are at a greater risk for CV-associated conditions than those that experience **late** menopause (Joakimsen *et al.*, 2000). Bone loss after menopause may be as high as 2% per year, causing dramatic effects on bone mass and the incidence of osteoporosis (Lane and Nydick, 1999). The estrogen loss associated with menopause is also thought to contribute to the development of Alzheimer's disease (Paganini-Hill and Henderson, 1996). Menopause can cause or exacerbate depression (Carandang *et al.*, 2000). Other

effects of menopause include an increased risk for vasomotor instability, hypertension, stroke, diabetes mellitus type 2, obesity, congestive heart failure, colon cancer, arthritis, and breast and ovarian cancer (Cutson and Meuleman, 2000; Masten and Gary, 1999; Sowers and LaPietra, 1995; Franceschi *et al.*, 2000).

CONSEQUENCES OF OVOTOXICITY

Certain environmental chemicals (i.e. ovotoxic substances) can destroy ovarian follicles. The consequences of this destruction vary depending on what type of follicle is targeted. If antral and preovulatory follicles are destroyed by a toxicant, an immediate loss of fertility results, however, this loss is temporary. Once the exposure stops and the chemical is eliminated from the body, cyclicity can resume as small follicles are recruited for development from the non-targeted primordial pool of follicles (Hirshfield, 1997).

On the other hand, if a chemical affects smaller primordial and primary follicles, the exposure may not affect fertility immediately. Rather, the non-replaceable pool of small follicles will be depleted at a quicker rate, causing premature ovarian failure (menopause) and permanent infertility (Hoyer and Sipes, 1996; Tilly, 1998; Hirshfield, 1997). Exposure to substances which destroy small follicles are of particular concern because the toxicity may go undetected for years since regular menstrual cycles continue. Since more and more women are delaying the start of their families to pursue a career, early loss of fertility is an important concern. Additionally, animal studies have shown that the destruction of primordial follicles enhances the development of ovarian

neoplasms (Hoyer and Sipes, 1996). Therefore exposure to chemicals that target small pre-antral follicles can cause precocious, irreversible loss of fertility as well as enhanced exposure to the health risks associated with menopause. Of additional concern are pregnant women who are exposed to ovotoxic chemicals. Animal studies have shown that *in utero* exposure can cause a reduced reproductive capacity in their unborn female pups since the developing primordial germ cells and oogonia are highly sensitive to destruction (Johannisson and Ocker, 1997; Tam and Liu, 1985; Mattison and Schulman, 1980).

TYPES OF OVOTOXIC CHEMICALS

Animal studies have demonstrated a variety of toxicants which target ovarian follicles for destruction. Chemotherapeutic drugs, while prolonging a cancer patient's survival, can cause sterility. Depending on the type and duration of treatment, up to 50% of women receiving chemotherapy will undergo premature ovarian failure (Bokemeyer *et al.*, 1994). The most widely used chemotherapy drugs are the alkylating drugs or nitrogen mustards (Balmer and Valley, 1997). These drugs include cyclophosphamide, procarbazine, and triethylene-melamine which have been determined to destroy primordial and primary follicles and cause premature menopause or ovarian failure in human and rodents (Familiaria *et al.*, 1993; Howell and Shalet, 1998; Plowchalk and Mattison, 1992; Shiromizu *et al.*, 1984; Cattanach, 1959). Additionally, mouse oocytes treated *in vitro* with therapeutic levels of another anti-cancer drug, doxorubicin, were

found to undergo apoptosis based on morphological (condensation, budding, and cellular fragmentation) and biochemical analysis (Perez *et al.*, 1997). While virtually no apoptosis was seen in control oocytes, greater than 60% of doxyrubicin treated oocytes were undergoing apoptosis as determined by morphology. *Bax*-deficient mice were resistant to doxyrubicin-induced oocyte destruction and mice treated with a caspase inhibitor were also rescued from this toxicity, further suggesting the role of apoptosis in this mechanism of follicle loss.

A class of compounds found in cigarette smoke called polycyclic aromatic hydrocarbons (PAHs) have also been implicated in ovarian toxicity. Women that smoke >20 cigarettes/day enter menopause 1-5 years earlier than nonsmoking women (Mattison *et al.*, 1989; Mattison, 1982). Smoking has also been associated with decreased fertility by up to 57-75% in smokers as compared to nonsmoking women (Baird and Wilcox, 1985). This human evidence suggests that chemicals in cigarette smoke destroy both small and large follicles and animals studies confirm this reasoning. 3-Methylcholantrene (MC), 7,12-dimethylbenz[a]anthracene (DMBA), and benzo[a]pyrene (BaP) are three types of PAHs found in cigarette smoke and their ability to destroy ovarian follicles of all sizes in rats and mice has been well characterized (Mattison, 1980; Borman *et al.*, 2000).

Environmental, industrial, and occupational chemicals are an additional source of ovotoxic chemicals. Among other reproductive effects, the industrial solvents, 2,2-bis (bromoethyl)-1,3-propanediol (BPD), ethylene glycol monomethyl ether (EGME), and EGME's metabolite, methoxyacetic acid (MAA), cause 33 to 92% follicle loss (Bolon *et*

al., 1997). Transplacental exposure to BPD also reduced follicles counts in the F_1 generation females. Exposure to 2-bromopropane (2-BP) has been shown to cause ovarian dysfunction in female factory workers (Yu *et al.*, 1999). Inhalation studies in rats have shown that 2-BP selectly destroys primordial follicles via apoptosis (Yu *et al.*, 1999). Oral exposure to hexachlorobenzene in monkeys, a persistent chlorinated chemical that has been detected in human ovarian follicular fluid, destroys primordial follicles (Jarrell *et al.*, 1993). Gavage studies in rats have shown that di-(2-ethylhexyl) phthalate (DEHP), a chemical used to add flexibility to plastics for food and medicine packaging, reduces antral follicle size and prevents ovulation (Davis *et al.*, 1994). Additionally, occupational exposure to chemotherapeutic drugs has been shown to reduce fertility in nurses and pharmacy personnel (Valanis *et al.*, 1997). 1,3-Butadiene, a common gas produced for use in plastics, flame retardants, insecticides, and rubber tires, is also found in automobile exhaust, cigarette smoke, and pollution (Morrow, 1990). Butadiene destroys preantral follicles in mice, but not rats, following repeated exposure (NTP, 1993). Its metabolite, butadiene diepoxide, however, targets pre-antral follicles in both rats and mice (Doerr *et al.*, 1996). Butadiene can dimerize to form vinylcyclohexene (VCH), a volatile industrial chemical. VCH and its epoxide metabolite, 4-vinylcyclohexene diepoxide, VCD, are also known to have toxic effects on ovarian follicles in laboratory animals.

VCH AND VCD

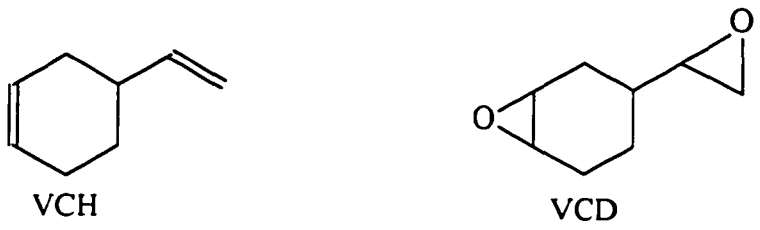


Figure 1.3. Chemical structures of VCD and VCH.

VCH is released as a byproduct from industrial manufacture involving butadiene and produced as an intermediate in styrene synthesis (IARC, 1994a; Haggin, 1994.). VCD is released as a byproduct of vinylnorbornene production and produced for use as a reactive diluent for other diepoxides. Additionally, VCD can be used for embedding biological tissues for electron microscopy (IARC, 1994b). VCH can also be converted to VCD via the cytochrome P450 enzymes (Smith *et al.*, 1990a). Human exposure to both of these chemicals is primarily through inhalation, however dermal contact is also a route of exposure for VCD (NTP, 1986; Maronpot, 1987; NTP, 1989). Following two years of exposure, VCH was carcinogenic and caused the induction of rare ovarian tumors in mice, but not rats (NTP, 1986). In 30 day studies, it was found that dosing with VCH caused severe follicle destruction in murine primordial and primary follicles (Smith *et al.*, 1990b). Within a year of receiving 30 daily doses of VCH, B6C3F_1 mice had undergone premature ovarian failure and were developing early signs of neoplastic changes (Hooser *et al.*, 1994). Additionally, it has been demonstrated that VCD can affect normal ovarian

development if exposures occur *in utero* (Grizzle *et al.*, 1994). The consequences of exposure to VCD in humans are unknown.

VCH causes follicular toxicity in only mice, while dosing with VCD causes follicle destruction in both rats and mice (Smith *et al.*, 1990b). VCH is converted to VCD in a two step process involving the epoxidation of both double bonds to epoxide groups. Cytochrome P450 enzymes can convert either double bond position to an epoxide, causing the formation of two monoepoxide intermediates, VCH-1,2-epoxide or VCH-7,8-epoxide. In mice, significantly more of the radiolabeled 1,2-epoxide was seen after VCH treatment as compared to rats (Smith *et al.*, 1990b). Differences in the P450 enzymes of rats and mice might explain this species difference, however the cytochrome P450 isoforms responsible for this conversion are not known yet. Both P450 2A and 2B are expressed in higher levels in mice than rats as measured by hepatic microsome immunoblotting (Smith *et al.*, 1990a). Murine hepatic metabolism of VCH to VCD was diminished if mice were treated with antibodies against cytochrome P4502A and 2B (Smith *et al.*, 1990a). However, rats treated with phenobarbital, a known inducer of P450 2A and 2B, did not show an increased ability to convert VCH to its monoepoxides (Smith *et al.*, 1990a). Therefore, P450 2A and 2B may not be responsible for the species differences between rats and mice. While the exact mechanism of conversion from VCH to VCD has not been determined, it is known that mice have a greater capacity to convert VCH to VCD than rats.

After 30 days, a 50% loss of preantral follicles was caused in mice dosed with 2.7 mmol/kg VCH, 0.5 mmol/kg VCH-1,2-epoxide, 0.7 mmol/kg VCH-7,8-epoxide, or 0.2

mmol/kg VCD. Therefore in mice, the ovotoxicity of VCD is greater than VCH-1,2-epoxide, which is greater than VCH-7,8-epoxide, which is greater than VCH. Rats cannot convert VCH to VCD and are not sensitive to VCH, but are sensitive to VCD. Taken together, these findings suggest that VCH must first be metabolized to VCD, the bioactive form of this ovotoxicant (Smith *et al.*, 1990c).

MECHANISMS OF VCD-INDUCED OVOTOXICITY

The cellular effects of VCD-induced ovotoxicity have been well characterized. Following 15 daily doses of VCD (80 mg/kg, i.p.), 50% of primordial and primary follicles are destroyed in the ovaries of immature rats (d28-42) (Smith *et al.*, 1990b, Flaws *et al.*, 1994a). A similar pattern of damage was seen in adult rats exposed to VCD for 30 days (Flaws *et al.*, 1994a). Morphological evaluation of ovaries from VCD-treated animals provide evidence that ovotoxicity is via apoptosis, rather than necrosis (Springer *et al.*, 1996c). After 10 daily doses of VCD, no follicle loss has occurred, but the morphological evidence of impending damage was seen. At that time, expression of mRNA encoding the cell death enhancer, *bax*, was increased as compared to control (Springer *et al.*, 1996b). Interestingly, following a single dose of VCD, the percent of healthy primary follicles was increased as compared to control and this was underscored 15 days later by an increase in the total number of primary follicles (Borman *et al.*, 1999). Accompanying this protective effect of a single dose of VCD was a change in the pattern of gene expression. That is, mRNA encoding *bax* in small preantral follicles was

decreased four hours after a single dose of VCD when compared with controls (Borman *et al.*, 1999). It has been proposed, therefore, that VCD causes ovotoxicity by accelerating the normal rate of atresia. Thus, following a single dose, VCD is protective and after repeated doses, VCD accelerates apoptosis to cause toxicity. These findings suggest that VCD is interacting in the normal pathway of atresia.

The molecular mechanisms of both atresia and VCD-induced ovotoxicity are still unknown. Since apoptosis is a regulated process, several pathways are likely to be altered prior to the point of no return, cell death. Upstream modulators in the cell may interact with VCD to signal apoptosis (McGee *et al.*, 1998; Hsu and Hsueh, 2000). These could include any number of factors which modulate atresia such as estradiol or growth factors. VCD could initiate an oxidative stress response or interact with an endogenous receptor in the follicle, which in turn might affect several cell death and cell survival factors. The Bcl-2 family of proto-oncogenes are known to be involved in the determination of cell fate in some tissues (Tsujimoto and Shimizu, 2000). This group of related proteins can form homo- and heterodimers and the specific configuration of these dimers is one of the ways these proteins determine cell survival versus cell death (Motyl, 1999). Members of this family include the cell survival genes *bcl-2* and *bcl-*X_L and the cell death enhancer genes *bax*, *bcl-*X_S, and *bad* (Hsu and Hseuh, 2000). The expression and localization of these proteins can shift the cell to favor either cell survival or cell death (apoptosis). For example, Bax/Bcl-2 heterodimers favor cell survival. An increase in Bax with no change in Bcl-2 can result in the increased formation of Bax/Bax homodimers and therefore, a shift to cell death. Additionally, the cellular localization of

these proteins is important. A translocation of Bax or Bad to the mitochondria where it could form or alter mitochondrial channels can also result in apoptosis (Hsu and Hseuh, 2000). The Bcl-2 family of proteins can cause a change in the mitochondrial membrane permeability by forming and regulating ion channels on the mitochondrial membrane (Gross *et al.*, 1999). It is postulated that there is a megachannel termed the permeability transition pore which is opened in the mitochondrial membrane during the early events of apoptosis. This channel connects the inner and outer membranes of the mitochondria and once opened, causes the collapse of the mitochondrial membrane potential, uncoupling of the respiratory chain, efflux of small molecules and proteins from the organelle, a loss of mitochondrial RNA and protein synthesis, release of calcium and GSH from the mitochondrial matrix, cessation of ATP synthesis, and hyperproduction of superoxide anion (Kroemer *et al.*, 1998). The end result is production of self-amplifying feedback loops since some of the products released from this organelle are also stimulators of the process.

In the mechanism of Bax-induced opening of the megachannel, the mitochondrion releases Cytochrome *c* from an intramitochondrial location, where it leaks into the cytosol and combines with other factors to activate downstream caspases (Cai *et al.*, 1998). Activation of these caspases is believed to bring the cell to the point of no return in this cell death pathway. Caspase-3 is a potent protease and destroys proteins throughout the cell. Additionally, Caspase-3 subsequently activates a DNA fragmentation factor, which in turn activates nucleases (Cai *et al.*, 1998).

Rats dosed daily with VCD for 10 or more days show an increased expression of the mRNA encoding *bax* as compared to control in isolated small pre-antral follicles (Springer *et al.*, 1996b; Borman *et al.*, 1999). After 15 days of daily dosing with VCD, the amount of Bad protein (pro-apoptotic) associated with the mitochondria and in the cytosol is increased and Bcl-X_L becomes translocated from the mitochondria to the cytosol (pro-apoptotic event; Hu *et al.*, 2001a). These events are accompanied by cytochrome *c* leakage into the cytosol (Hu *et al.*, 2001a). An increase in amount of both active Caspase-3 expression and activity has also been shown following 15 days of VCD treatment in small follicles as compared to control (Hu *et al.*, 2001b). All of these data support that VCD enhances apoptosis in the targeted small pre-antral follicles.

While many of the late apoptotic events of VCD-induced ovotoxicity have been determined, the early events in this signaling pathway are largely unknown. VCD may initiate an oxidative stress response. This might involve alterations in the endogenous cellular antioxidant, glutathione (GSH). In this manner, VCD could deplete cellular GSH through conjugation and formation of a GSH-VCD metabolite. This would then lead to an accumulation of reactive oxygen species and eventually the initiation of apoptosis. An additional and/or alternative line of thought is that VCD initiates the apoptotic signal by interacting with an endogenous receptor such as the estrogen or aryl hydrocarbon receptor in the follicular cells.

ARYL HYDROCARBON RECEPTOR

The aryl hydrocarbon receptor (AhR) or xenobiotic receptor has been shown to be involved with apoptosis. The AhR has been shown to bind a wide variety of environmental chemicals including halogenated aromatic hydrocarbons, biphenyls, and dibenzofurans (Denison and Heath-Pagliuso, 1998). The most potent ligand of the AhR is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD; dioxin) and has been found to be one of the most toxic (to humans and other mammals) man-made chemicals known (Denison and Heath-Pagliuso, 1998). Additionally, the polycyclic aromatic hydrocarbons (PAHs) are known to signal via the AhR. To date, the endogenous ligand of the AhR is unknown.

In animals and humans, exposure to dioxin causes varied toxicities. Physiological effects induced by dioxin via the AhR include immunotoxicity, chloracne, hyperkeratosis, lethality, tumor promotion, porphyria, wasting syndrome, hepatotoxicity, teratogenicity (cleft palate), and endocrine disruption (Denison and Heath-Pagliuso, 1998). Rats exposed to TCDD *in utero* have vaginal clefting, vaginal thread formation, cleft clitoris, decreased ovarian weight, and decreased numbers of corpus lutea and antral follicles (Gray and Ostby, 1995; Flaws *et al.*, 1997; Silbergeld and Mattison, 1987; Heimler *et al.*, 1998a). Many chemicals, like PAHs, work through the AhR and destroy follicles (Mattison, 1980). This ovotoxicity can be blocked by treatment with the AhR antagonist, α-napthoflavone (Mattison and Thorgeirsson, 1979).

Expression of the AhR has been found in virtually all mammalian and a number of nonmammalian vertebrate species (Schrenk, 1998; Hahn, 1998). It is a member of the PAS (period-arnt-singleminded) family of basic helix-loop-helix proteins (Burbach *et al.*, 1992; Ema *et al.*, 1992, Gu *et al.*, 2000). Proteins with PAS domains play a role in detection of and adaptation to environmental changes caused by xenobiotics, hypoxia, and light (circadian response pathway; Gu *et al.*, 2000). The AhR protein harbors domains for both DNA binding and protein dimerization and is well conserved in mammals, however highly polymorphic, especially compared to other nuclear receptors (Schmidt and Bradfield, 1996; Gonzalez, 1995). The polymorphisms result in different sized AhR proteins from 95-105 kDa and a variety of responsiveness (Schmidt and Bradfield, 1996). Based on treatment with PAHs, some strains of mice are extremely responsive to these AhR ligands, while others are virtually non-responsive (Nebert and Gelboin, 1969). However, even in non-responsive strains, TCDD is able to elicit similar AhR responses, at 10-100 fold higher doses than in responsive strains (Poland *et al.*, 1974). Genes known to be regulated by the AhR include a wide variety of metabolizing enzymes including cytochrome *P450 1A1/2*, *P450 1B1*, glutathione S-transferase (*GST*) *Ya1* and *Ya2*, NAD(P)H quinone oxidoreductase (*Nqo1*), aldehyde dehydrogenase 3 (*Aldh3a1*), uridinediphosphate-glucuronosyl transferase 1a6 (*UDPGT 1a6*), γ-aminolevulinic acid synthase, prostaglandin endoperoxide H synthase 2, and interleukin 1β (Safe, 1990; Safe, 1995; Devito and Birnbaum, 1994; and Hankinson, 1995).

The AhR normally resides in the cytosol bound to a HSP90 dimer and ARA9 (AIP1, AhR interacting protein; Carver *et al.*, 1998). These regulatory proteins maintain

the AhR in a ligand binding position, thereby increasing the number of functional receptors in the cytosolic compartment (Gu *et al.*, 2000; Carver *et al.*, 1994; LaPres *et al.*, 2000). Upon ligand binding, the AhR dissociates from both the HSP90s and ARA9, translocates to the nucleus, and interacts with the AhR nuclear transporter (ARNT) (Hoffman *et al.*, 1991). The AhR-ARNT heterodimer then binds the xenobiotic response element of the DNA promoter region (XRE, sequence =TNGCGTG) and initiates transcription (Gu *et al.*, 2000). Activation of the XRE can result in negative or positive transcriptional regulation, however a negative response element (NRE) has been described upstream of the [*Ah*] battery of genes (Nebert *et al.*, 2000). Promoter-analysis studies have proposed the existance of NREs and a 21-base pair inverted repeat has been found upstream of the *CYP1A1* gene in human, mice, and rats as well as in rat and mouse *ALDH3A1* genes (Gonzalez and Nebert, 1985; Piechochki and Hines, 1998; Xie *et al.*, 1996; Nebert *et al.*, 2000). Taken together, transcription regulation by the AhR involves both up-regulation of gene expression by its XRE and down-regulation of genes by its XRE and NRE.

At the cellular level, the AhR is known to play a role in oxidative stress response, cell cycle control, and apoptosis (Nebert *et al.*, 2000). As part of a cell's response to oxidative stress, expression of *Nqo1*, *Aldh3a1*, *Ugt1a6*, and *GST Ya1/Ya2* is up-regulated. These genes work to decrease reactive oxygen species and eliminate toxicants from the cell. However, at the G1/S boundary of the cell cycle, oxidative stress can flip the switch to apoptosis rather than continuation of the cell cycle (Nebert *et al.*, 2000). In the mouse, the *bax* gene promoter contains two consensus XRE sequences suggesting a direct role of

the AhR in apoptosis that does not require an oxidative stress response (Robles *et al.*, 2000). Several ligands of the AhR have been shown to induce apoptosis in their target tissues, however it has not been determined if this was initiated by an oxidative stress response (Near *et al.*, 1999; Zaher *et al.*, 1998; Lutz *et al.*, 1998).

The most well understood function of the AhR is its interaction with xenobiotics. The physiological roles of the AhR have been hard to determine, therefore AhR deficient mice (AhRKO) were created. Two independent lines of AhR null mice have been made to determine the endogenous role(s) of the AhR (Fernandez-Salguero *et al.*, 1995; Schmidt *et al.*, 1996). AhRKO mice are characterized by slow whole body growth, decreased liver weight, liver abnormalities, and immune system problems (Lahvis and Bradfield, 1998). Both male and female AhRKO mice are fertile, but female AhRKO have fewer total and live offspring at birth than the wild type (Abbott *et al.*, 1999). After gestation, AhRKO dams and pups had a reduced ability to survive during the lactational period as compared to wild-type animals (Abbott *et al.*, 1999).

Currently, two studies have been completed in AhRKO mice which have examined ovarian follicles. Robles *et al.* (2000) stained wild type and AhRKO mouse ovaries for AhR protein expression. Wild type animals had extensive staining in granulosa cells and oocytes of all sizes of follicles including primordial while no staining was seen in the AhRKO mice. Robles *et al.* (2000) also determined, by follicle counting, that there was a decrease in the appearance of atretic primordial follicles in AhRKO mice as compared to wild type litter mates. They concluded that inactivation of the *AhR*-regulated gene leads to increased rates of germ cell survival (decreased atresia) and an

enlarged primordial follicle reserve due to the enhanced survival of developing germ cells during prenatal gametogenesis. In another study, Benedict *et al.* (2000) found that AhRKO mice had similar numbers of germ cells on gestational day 18. However, by postnatal day 2-3, AhRKO had more fully formed primordial follicles and fewer single germ cells as compared to wild type. By postnatal day 8 and 32-35, there were no differences in follicle numbers between wild type and AhRKO. At day 53, AhRKO mice had a reduction in the number of antral follicles. Benedict *et al.* (2000) concluded in this study that the AhR plays a role in primordial follicle formation and antral follicle regulation.

AhRKO mice appear to have altered follicular development and atresia. During prenatal life, the AhR appears to suppress the normal rate of atresia, while in mature animals, the AhR appears to suppress growth into mature stage follicles (Benedict *et al.*, 2001). Taken together, the AhR appears to play differing roles in normal follicular dynamics and atresia based on the developmental stage of the follicle. Additionally, the AhR is known to signal interleukin-1β (Il-1β) transcription. As described earlier, Il-1β is also known to regulate atresia of large follicles. This provides further evidence that the AhR not only mediates xenobiotic signaling, but also plays a physiological role in the ovary.

ESTRADIOL AND ESTROGEN RECEPTORS

The estrogen receptor (ER) is a member of the steroid hormone receptor family, a large superfamily (>70 members) of nuclear receptors that function as ligand-activated transcription factors (Katzenellenbogen, 1996). Estrogen receptors shuttle between the cytoplasm and nucleus, however in steady-state conditions, are primarily found in the nucleus (Parker, 1995). In the unliganded state, the ER is complexed with 2 molecules of heat shock protein 90 (HSP90; Carson-Jurica *et al.*, 1990). HSP90 is a highly conserved, abundant cytosolic protein that is thought to stabilize receptors by placing them in a favorable position for ligand-binding (Carson-Jurica *et al.*, 1990). While HSP90 is bound to the ER protein, DNA cannot bind the receptor. Upon ligand binding to the ER, the HSP90 proteins dissociate from the receptor, exposing the DNA-binding domain. The ER-ligand complex homodimerizes with another ER-ligand complex and the resulting dimer then binds with high affinity to an estrogen response element (ERE; Parker, 1995). EREs are found in the promoter region of genes and are characterized by an inverted repeat of the sequence A/GGTCA separated by 3 base pairs (Parker *et al.*, 1995). Upon DNA binding, the ER interacts with receptor-interacting proteins (RIPs; Parker, 1998). These proteins are thought to stabilize binding of the basal transcription factors either directly or indirectly to a region 30 bp downstream from the TATA box (Parker, 1995). Assembly of the pre-initiation complex stimulates RNA polymerase II to begin transcription (Parker, 1995).

ERs are characterized by several functional domains and structural similarities, with distinct regions labeled A-F for ligand-binding, transcription activation, and DNA-binding (Krust *et al.*, 1986; Katzenellenbogen, 1996; Carson-Jurica *et al.*, 1990; Parker, 1995). The A/B domain, located at the N-terminal side of the ER protein, is involved in transactivation by directly interacting with components of the core transcriptional machinery or with coactivators that mediate signalling to downstream proteins (Enmark and Gustafsson, 1999). The C domain is a highly conserved DNA-binding domain and contains two zinc fingers, which are involved in specific DNA binding and receptor dimerization (Enmark and Gustafsson, 1999). The D domain confers flexibility and a "hinge" between the DNA- and ligand-binding domain. The D domain has been shown to influence DNA binding properties and may also serve as an anchor for some corepressor proteins (Enmark and Gustafsson, 1999). The sites for ligand binding, receptor dimerization, nuclear localization, and interactions with transcription coactivators and corepressors occur in the E domain (Enmark and Gustafsson, 1999). The ER is able to interact with a wide variety of structurally different chemicals, a unique characteristic for the steroid family of receptors (Kuiper *et al.*, 1998). Finally, on the C-terminal end, the F domain is known to contribute to the transactivational capacity of the estrogen receptor and influence agonist/antagonist balance and potency, however most of the function of this region is unknown (Katzenellenbogen, 1996; Enmark and Gustafsson, 1999).

The classical estrogen receptor was discovered in 1958 by Elwood Jensen and cloned in 1986 (Greene *et al.*, 1986). For many years, it was thought there was only one

ER isoform and that the ER was required for viability. In 1994, a male patient was identified as lacking a functional ER (Smith *et al.*, 1994d). This suggested that the classical ER was not required for life. Additionally, the estrogen receptor knock-out (ERKO) mouse was developed in 1993, and in some tissues, specific estrogen binding could be measured (Lubahn *et al.*, 1993). In 1996, a second estrogen receptor isoform was cloned and the original ER became designated ERα and the new one, ERβ (Kuiper *et al.*, 1996). ERα and ERβ are distinct gene products encoded on different chromosomes (ERα = chromosome 6; ERβ = chromosome 14). ERβ is 95% homologous to ERα in the DNA-binding domain and 55% homologous in the ligand-binding domain (Enmark and Gustafsson, 1999; Gustafsson, 1999). Domains A/B and F are truncated in ERβ as compared to ERα (Domain A/B = 45% shorter, Domain F = 45% shorter; Kuiper and Gustafsson, 1997). Homology between the two receptors is quite low in the ligand-binding domain. In fact, in that region, ERα and ERβ are no more similar than the glucocorticoid and progesterone receptors are to each other (Enmark and Gustafsson, 1999). This difference between ERα and ERβ protein sequences therefore suggests that ligands can be specific for one ER isoform. Collectively, these may explain how some ligands act as agonists in one isoform and antagonists in the other. ERα and ERβ show differential tissue distribution with ERα highly expressed in uterus, testis, pituitary, ovary, kidney, epididymis and ERβ highly expressed in brain, prostate, ovary, lung, bladder, and epididymis (Kuiper *et al.*, 1997). Additionally, it has been found in mice that ERα and ERβ can form heterodimers to mediate ER signaling (Pettersson *et al.*,

1997). Therefore, in cells that express both isoforms of the ER, many ER signaling pathways can be used and may result in different patterns than the classical ER homodimer pathway.

Preovulatory and pre-antral follicles were not known to express the classical estrogen receptor, ERα, and therefore it was assumed for many years that follicular development was 17β-estradiol independent. Since its discovery, ERβ has been reported to be expressed in the granulosa cells of preovulatory and pre-antral ovarian follicles (Kuiper *et al.*, 1996; Ahluwalia *et al.*, 1998). The ratio between ERα and ERβ in the ovary is about 1:9 (Gustafsson, 1999). This suggests potential roles for 17β-estradiol (E_2) in follicular development and cell fate, however the specific actions of E_2 are unknown (Enmark *et al.*, 1997). Several types of knock-mice have been developed to try and discern the roles of E_2, ERα and ERβ. Aromatase, *cyp19*, is the enzyme which converts testosterone to estradiol. Aromatase-deficient mice (ArKO) have no detectable circulating estradiol (Fisher *et al.*, 1998). These mice are infertile due to a defect in ovulation, however they display follicular development to the antral stage. The authors report in female ArKO mice that there were many smaller follicles that appeared atretic, however this observation was not compared to wild-type litter mates (Fisher *et al.*, 1998). The ovaries of ERα-deficient mice (ERKO) are characterized by cystic haemorrhagic follicles and a lack of corpora lutea (Lubahn *et al.*, 1993). Female ERKO mice exhibit elevated serum 17β-estradiol, LH, and gonadotropin receptor expression, and males and females are both infertile (Korach, 2000). Female ERβ knock-out mice (BERKO) have reduced fertility due to a block in the last step of follicular development prior to

ovulation, and have few or no corpora lutea (Krege *et al.*, 1998). Taken together, observations in these transgenic models suggest that ERα and ERβ have different physiological roles in follicular development.

Due to differences in the ligand-binding domains of ERα and ERβ, the two ERs respond differently to various ligands. ERβ appears to be more sensitive than ERα to the endocrine disruptors classifieds as xenoestrogens. These endocrine disruptors include: phytoestrogens, like genistein and other flavonoids; lignans; coumestans; and environmental contaminants like polychlorinated biphenyls (PCBs) and methoxychlor (Kuiper *et al.*, 1998). In countries with a high intake of phytoestrogens, there is a significantly lower incidence of breast and prostate cancer, suggesting that these xenoestrogens prevent cancer (Messina *et al.*, 1994). Dietary soy, rich in phytoestrogens, has been demonstrated to prevent chemically and irradiation-induced mammary tumors in mice (Messina *et al.*, 1994). Since ERβ appears to play a more important role in binding of xenoestrogens, ERβ may also play a role in suppressing cell proliferation and cancer prevention. It appears that tamoxifen and some phytoestrogens work as antioxidants, therefore ERβ may control some antioxidant-regulated genes (Gustafsson, 1999). It was found in MCF-7 breast cancer cells that ERβ can interact with an antioxidant response element (ARE, sequence=TGACNNNGC) in the quinone reductase gene (Nebert *et al.*, 2000; Montano *et al.*, 1998). If ERβ interacts with an antioxidant pathway, then activation of ERβ would reduce concentrations of free radicals and reactive oxygen species in the cell, protecting it against cell damage and demise.

A role for E_2, the endogenous ligand of ERα and ERβ, in the control of apoptosis is not a novel concept. E_2 has been found to shift expression of the *bcl-2* gene family to suppress apoptosis in the corpus luteum of rabbits (Goodman *et al.*, 1998). Human endothelial cell apoptosis is inhibited by ER-mediated E_2 treatment (Spyridopoulos *et al.*, 1997). E_2 decreases apoptosis of peripheral blood mononuclear cells in women with normal menstrual cycles (Evans *et al.*, 1997). E_2 has also been found to be neuroprotective in the brain by inhibiting apoptosis and increasing *bcl-2* mRNA expression in several neuronal populations (Garcia-Segura *et al.*, 1998). Estrogen upregulates *bcl-2* mRNA levels in human breast cancer cell lines and protects MCF-7 cells from apoptosis (Wang and Phang, 1995). The death of human bone osteocytes via apoptosis accompanied estrogen withdrawal (Tomkinson *et al.*, 1997). *In vitro* treatment with E_2 significantly lowered the percent of apoptotic cardiac myocytes and reduced Caspase-3 activity (Pelzer *et al.*, 2000). The tumor suppressor p53, another gene involved with the control of apoptosis, is a negative regulator of estrogen receptor signaling pathways (Yu *et al.*, 1997). The estrogen receptor, a transcription factor, can also interact with other transcription factors like p53, to produce transcription factor "cross-talk" in many cellular processes.

INTERACTIONS BETWEEN ER AND AHR

The ER and AhR are both transcription factors that are known to interact with one another. Agonists of the AhR are known to act as endocrine disruptors and antiestrogens

(Safe *et al.*, 1998). These effects are seen in opposing carcinogenic abilities of AhR ligands. While TCDD is known to induce hepatocellular and squamous carcinoma in animal models and cell culture, it also reduces the risk for mammary, uterine, adrenal, and pituitary tumors in female rats (NTP, 1982; Vessey *et al.*, 1983). Therefore, AhR ligands exert antiestrogenic affects on estrogen dependent cancers (Kharat and Saatcioglu, 1996). Cigarette smoke contains PAHs, known ligands of the AhR. Women that smoke have been found to have a reduced risk for endometrial cancer, an estrogen-sensitive cancer (Brinton *et al.*, 1993). Therefore, AhR ligands act as antiestrogens in tumorigenesis.

Besides cancer related responses, TCDD and related compounds inhibit several E_2-induced responses including uterine weight increase, peroxidase activity, progesterone receptor binding, extracellular plasminogen activator activity, epidermal growth factor (EGF) receptor binding, and *fos* and *EGF* receptor mRNA levels (Safe, 1995; Gierthy *et al.*, 1987). TCDD has been shown to inhibit other E_2-induced phenomena including lactate dehydrogenase activity and *prolactin receptor* and *heat shock protein 27* mRNA levels (Safe *et al.*, 1998).

Molecular mechanisms of how liganded AhR is able to oppose liganded ER signaling are not clear and likely involve many levels of inhibition. AhR ligands have not been found to bind to estrogen or progesterone receptors directly (Zacharewski *et al.*, 1991). There are mixed reports whether AhR ligands affect ovarian steroidogenesis (Safe *et al.*, 1998). *In vitro* studies of human luteinized granulosa cells have shown that either E_2 or progesterone, but not both, is decreased following exposure to TCDD (Heimler *et*

al., 1998b; Enan *et al.*, 1996; Moran *et al.*, 1997). In one study, granulosa cells recovered their E_2 secretion to control levels upon addition of androstenedione, suggesting that the TCDD-induced defect results in the depletion of an androstenedione precursor (Heimler *et al.*, 1998a). However, TCDD also induces apoptosis in these steroidogenic granulosa cells. Therefore, the change in steroid secretion could have been due to loss of viability in the granulosa cells, rather than a direct effect on steroidogenesis itself (Heimler *et al.*, 1998b). In an *in vitro* study using granulosa and theca-interstitial cells collected from super-ovulated immature hypophysectomized rats, TCDD had no affect on granulosa or theca-interstitial cell steriodogenesis (Son *et al.*, 1999). Therefore Son *et al.* (1999) concluded that TCDD-induced ovulation defects are due to something other than a disruption of follicular steroidogenesis. Whether or not the AhR affects steroidogenesis directly, liganded-AhR induces *cyp 1A1, cyp 1A2, and cyp 1B1* expression. This serves to enhance oxidative metabolism of E_2 to various catechols and reduce E_2 action by shortening its half-life (Martucci and Fishman, 1993; Safe *et al.*, 1998).

One of hypotheses of ER and AhR "cross talk" is that thought to occur by binding of the AhR/ARNT heterodimer to imperfect ERE sites or to XREs adjacent or overlapping to EREs (Klinge *et al.*, 1999; Duan *et al.*, 1999; Kharat and Saatcioglu, 1996). Therefore, liganded AhR would bind DNA (either at its own XRE or to an imperfect ERE) and thereby block ER binding to its ERE.

Another proposed mechanism of AhR mediated reduction in ER-mediated transcription activation is via interaction with estrogen receptor coactivator/corepressor proteins. Transcriptional activation by the ER and other members of the nuclear receptor

superfamily requires interaction with other nuclear proteins including coactivator and corepressor proteins (Jenster, 1998; Nguyen, 1999). The coactivator, ER associating protein 140 (ERAP 140), and the corepressor, silencing mediator for retinoic acid and thyroid hormone receptor (SMRT), were found to interact with AhR/ARNT dimers, as well as increase (ERAP 140) or decrease (SMRT) AhR/ARNT binding to XREs (Nguyen, 1999). Additionally, the corepressor COUP-TF, an orphan nuclear receptor highly expressed in estrogen target tissues, is known to repress ER-regulated gene expression and has recently been found to bind XREs as well interact with the AhR protein itself (Klinge *et al.*, 2000). Therefore, AhR-mediated antiestrogenic responses could be related to competition between the ER and AhR signaling pathways for coactivators and corepressors (Nguyen, 1999).

The antiestrogenic properties of the AhR could also be in part due to a down regulation of ER protein. In guinea pigs, TCDD decreased basal hepatic ER levels, and in MCF-7 and Hepa 1c1c7 cells, TCDD reduced ER protein (Safe *et al.*, 1991; Hruska and Olson, 1989; Zacharewcki *et al.*, 1991). mRNA encoding *ERα* has also been found to be decreased following TCDD exposure as well in ovary, uterus, lung, and liver (Tian *et al.*, 1998; DeVito *et al.*, 1990; Colella and Gallo, 1993). The estrogen receptor gene has been found to contain five partial XRE sequences (GCGTG, core sequence) and one full length XRE sequence that binds the AhR (White and Gasiewicz, 1993). The core pentanucleotide sequence GCGTG allows AhR binding to DNA, however it is not sufficient for transactivation (Denison *et al.*, 1998). Functional inhibitory partial XREs have also been reported in the estrogen-responsive cathepsin D and pS2 genes (Krishnan

et al., 1995; Gillesby *et al.*, 1997). The full length XRE in the ER promoter region has been found to specifically bind activated AhR with high affinity *in vitro* (White and Gasiewicz, 1993). Therefore, the endocrine disrupting abilities of AhR ligands could be through a decrease in ER expression. However, TCDD is still antiestrogenic in cells that constitutively express the ER due to an expression vector that cannot be downregulated (Kharat and Saatcioglu, 1996). Thus, the antiestrogenic properties of TCDD and other ligands of the AhR are not merely due to a decrease in ER expression.

While most studies have shown that AhR ligands act as antiestrogens, there is evidence that E_2 can interfere with AhR signaling as well. *In vitro* E_2 treatment in cell lines reduces TCDD-induced *cyp 1A1* mRNA expression as well as CYP 1A1 activity (Ricci *et al.*, 1999; Jeong and Lee, 1998; Kharat and Saatcioglu, 1996). Kharat and Saatcioglue (1996) found that liganded ER interfered with AhR binding to its response element. Ricci *et al.* (1999) found that E_2 did not prevent AhR binding to its XRE, rather that E_2 appeared to squelch available nuclear factor-1, a transcription factor known to interact with both ERs and AhRs. However Jeong and Lee (1998) found that E_2 did prevent AhR binding to its XRE by preventing AhR transformation into a form able to bind DNA. They concluded that E_2 acts as a noncompetitive antagonist to the AhR.

SPECIFIC AIMS

The following studies have been designed to investigate the role of estrogen receptor and aryl hydrocarbon receptor in VCD-induced ovotoxicity in rats. If

experimental evidence shows that VCD interacts with the ER and/or AhR, then it is likely that there is interaction between the two receptors as well. Subsequently, these studies will also examine potential interactions of these two pathways specifically involving HSP90. Therefore, the studies proposed here will test the hypothesis that: *VCD-induced ovotoxicity involves a nuclear receptor-mediated signaling pathway that leads to increased atresia.* To address this hypothesis, the following specific aims were established:

Specific Aim 1: To characterize the physiological role of the aryl hydrocarbon receptor in VCD-induced ovotoxicity. The AhR is thought to play a role in follicular development and atresia. Therefore its presence will be verified in small preantral follicles. The possible role of the AhR in VCD-induced ovotoxicity will also be examined using AhR antagonists and AhR deficient mice. The regulation of the AhR by VCD and some of the genes activated by the AhR will be investigated as well as the effect of VCD on AhR transactivation to DNA in the nucleus. (Chapter 2)

Specific Aim 2: To characterize the physiological role of the estrogen receptor in VCD-induced ovotoxicity. Circulating E_2 increases with the onset of sexual maturity in females. At this time there is also a dramatic decrease in the rate of atresia in the ovary. E_2 has been shown to inhibit apoptosis in large follicles, so this inverse correlation suggests a role of E_2 in the control of atresia. Since VCD is thought to enhance the

natural process of atresia, this suggests that E_2 could also be involved in VCD-induced ovotoxicity. (Chapter 3)

Specific Aim 3: To characterize the physiological role of heat shock protein 90 in VCD-induced ovotoxicity. HSP90 has been implicated to play a role in toxin-induced stress, ER and AhR signaling, as well as ER and AhR receptor interactions. The effect of an HSP90-function inhibitor on follicle numbers will be assessed following treatment with VCD. The localization of HSP90 protein and quantity of *HSP90* mRNA and protein will be analyzed in follicles following treatment with VCD and other receptor analogs. (Chapter 4)

CHAPTER 2

ROLE OF THE ARYL HYDROCARBON RECEPTOR IN 4-VINYLCYCLOHEXENE DIEPOXIDE-INDUCED OVARIAN FOLLICLE LOSS IN FISCHER-344 RATS

ABSTRACT

Repeated dosing with the occupational chemical 4-vinylcyclohexene diepoxide (VCD) selectively depletes small pre-antral (primordial and primary) follicles in the ovaries of rats and mice. Although this toxicity is caused via induction of apoptosis, the signaling mechanism that initiates this loss is not known. The aryl hydrocarbon receptor (AhR) plays a role in mediating the effects of a number of xenobiotics via regulation of expression of metabolic enzymes and induction of apoptosis. Therefore, this research was designed to examine the potential role of the AhR in VCD-induced ovotoxicity. Female F344 rats were dosed daily with either vehicle control, VCD (80 mg/kg), and/or the AhR antagonist alpha-naphthoflavone (ANF; 80 mg/kg) for 15 days. Four hours after the final dose, ovaries were collected and prepared for histological evaluation (N=7-9/group) or dissociated for isolation of small pre-antral follicles (N=3-4) and measurement of caspase-3-like activity, a biochemical marker of apoptosis. VCD alone caused a 60% reduction ($p<0.05$) in primordial and primary follicles. Concurrent dosing with ANF protected primordial and primary follicles from VCD-induced loss. Treatment with ANF alone resulted in a greater ($p<0.05$) number of these follicles, as compared to control. VCD dosing increased ($p<0.05$) caspase-3-like activity while concurrent treatment with ANF and VCD restored caspase-3-like activity to control levels. Dosing

with ANF alone reduced ($p<0.05$) caspase-3-like levels to below control. Expression of both AhR mRNA and protein was seen in VCD-targeted small pre-antral follicles and repeated dosing with VCD increased expression of *AhR* mRNA as compared to control. VCD dosing did not induce expression of CYP1A1 protein or *GST Ya1* or *Ya2* mRNA. To investigate the requirement of the AhR in mediating VCD-induced follicle loss in mice and rats, AhR null (-/-) C57BL/6 mice were dosed daily with vehicle control or VCD (80 mg/kg). As with AhR-intact mice and rats, VCD induced a 70% follicle loss ($p<0.05$) in AhR-null mice. These results support that the AhR is involved in VCD-induced follicle loss, however, it is not required. Therefore, while VCD does modulate expression of the *AhR*, it does not need the AhR to mediate its toxicity.

INTRODUCTION

Repeated daily dosing with the occupational chemical 4-vinylcyclohexene diepoxide selectively depletes small pre-antral follicles (primordial and primary) in the ovaries of rats and mice (Borman *et al.*, 1999; Borman *et al.*, 2000; Kao *et al.*, 1999; Springer *et al.*, 1996a). Female mammals are born with a finite number of these small pre-antral follicles, such that when these follicles become depleted, ovarian failure (menopause in women) occurs (Hirshfield, 1991a). Since primordial follicles cannot be regenerated, exposure to toxicants that target these immature follicles can cause premature ovarian failure in laboratory animals. In women, exposure to such toxicants may be associated with an early age at menopause, a condition associated with health risks.

Previous studies have demonstrated that VCD causes follicle depletion by programmed cell death, apoptosis, and accelerates the natural process of atresia (Borman *et al.*, 1999; Springer *et al.*, 1996a). However, the exact mechanisms that initiate VCD-induced follicle loss are unknown. Because VCD is specific for small pre-antral follicles (primordial and primary) and appears to alter a natural process in the ovary, VCD has been used as a model compound to study toxicants which target small pre-antral follicles, as well as a tool to elucidate the atretic signaling pathway.

The aryl hydrocarbon or xenobiotic receptor (AhR), a transcription factor, binds many structurally diverse toxicants, modulates transcription of many genes involved in xenobiotic metabolism, and regulates cell cycle, apoptosis, and oxidative stress (Denison and Heath-Pagliuso, 1998; Nebert *et al.*, 2000). To date, no known endogenous ligand has been identified for this receptor. The AhR is expressed in the ovary, and has been found to mediate toxicity of other xenobiotics which target ovarian follicles. Polycyclic aromatic hydrocarbons (PAH) destroy ovarian follicles in rats and mice (Borman *et al.*, 2000; Mattison and Thorgeirsson, 1979). This toxicity is thought to be mediated via the AhR. This destruction is accompanied by an increase in ovarian aryl hydrocarbon hydroxylase activity (CYP 1A1) and this PAH-induced follicle loss is prevented by concurrent treatment with the AhR antagonist α-naphthoflavone (ANF; Mattison and Thorgeirsson, 1978; Mattison and Thorgeirsson, 1979).

Recent studies from AhR-deficient mice (AhRKO) suggest an endogenous role of the AhR in the ovary. Benedict *et al.* (2000) found that on postnatal day 2-3, AhR -/- (AhRKO) mice contain more fully formed primordial follicles and less non-follicular

associated single germ cells than wild-type. However, by d53, AhRKO mice had fewer antral follicles than wild-type. In another study, Robles *et al.* (2000) reported that d4 AhR -/- mice contain twice as many primordial follicles as AhR +/+ mice. This was attributed to a reduction in germ cell attrition during prenatal gametogenesis. Therefore, the AhR appears to play a role in follicle formation and atresia in the mouse. These findings provide evidence that in addition to mediating xenobiotic signaling in many tissues, the AhR also plays a physiological role by regulating follicular dynamics in the ovary, however the specific details of this role remain to be determined. VCD is also known to accelerate the physiological process of atresia in the ovary (Borman *et al.*, 1999). Whether VCD and the AhR modulate ovarian follicles using similar pathways is not known. Therefore, this study was designed to investigate a possible role of the AhR in VCD-induced follicle loss.

METHODS

See Appendix B.

RESULTS

Following 15 daily doses of rats with VCD, there was a significant reduction of ovarian primordial and primary follicles compared with controls ($p<0.05$; Figure 2.1). After treatment with the high dose of the AhR antagonist ANF (80 mg/kg), the number of primordial and primary follicles was greater than in controls ($p<0.05$; Figure 2.1). There was no difference in the number of primordial or primary follicles between control and

combined treatment with the high dose of ANF and VCD. A dose related effect was seen by co-treatment with the low dose of ANF (20 mg/kg) and VCD with remaining primordial and primary follicles lower ($p<0.05$) than controls, but greater ($p<0.05$) than VCD-treatment (Figure 2.1). Secondary follicle numbers were not affected by any treatment.

In order to examine the effect of an AhR antagonist on normal and VCD-induced apoptosis, caspase-3 activity was measured in isolated small pre-antral follicles. Repeated daily dosing with VCD caused an increase ($p<0.05$) in caspase-3 activity as compared to control (Figure 2.2). This effect was negated by co-administration of the high dose of ANF and VCD. Treatment with the high dose of ANF alone showed a reduction ($p<0.05$) in caspase-3-like activity as compared to control. For comparison between follicle loss and caspase activities, primary follicle counts are also shown (Figure 2.2). Secondary follicle results are presented in Appendix A.1, Figure A.1.

To verify gene expression of the *AhR* in VCD-targeted small pre-antral follicles, RT-PCR was used. Expression of mRNA encoding the *AhR* was seen in both isolated fraction 1 (containing VCD-targeted primordial and primary follicles) and fraction 2 (non-targeted) follicles. Furthermore, repeated dosing with VCD increased ($p<0.05$) mRNA encoding the *AhR* in VCD-targeted fraction 1 follicles as compared to control (Figure 2.3). VCD dosing had no affect on *AhR* mRNA expression in non-targeted fraction 2 follicles (Figure 2.3).

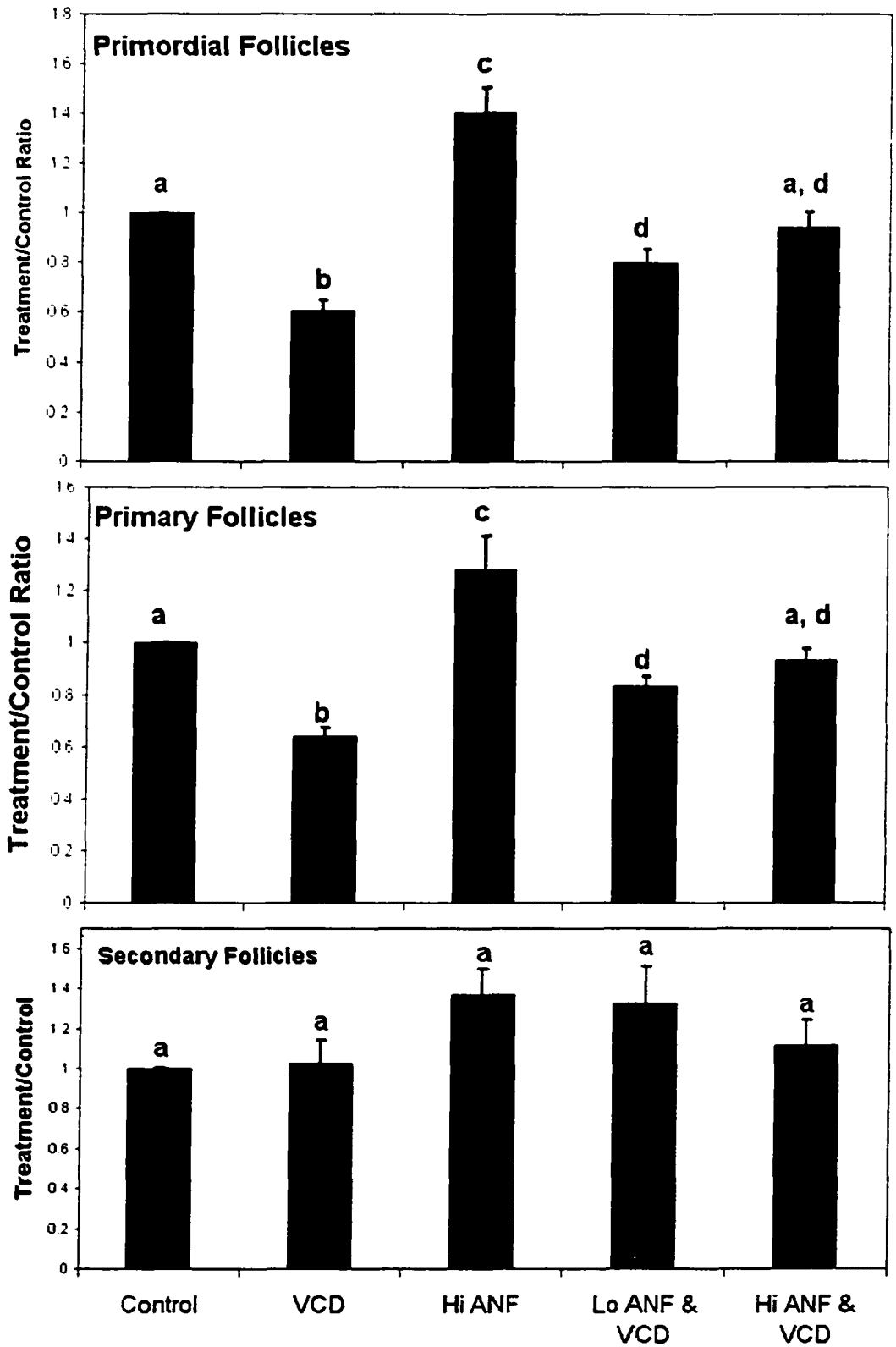
Distribution of the AhR protein was examined in small pre-antral follicles using immunohistochemistry and confocal microscopy. AhR protein was visualized in VCD-

targeted primordial and primary follicles (Figure 2.4). When images were merged, co-localization of the red stain for the AhR and the green nuclear stain YOYO-1 appeared yellow, demonstrating that the AhR is located in the nucleus of granulosa cells in all sizes of follicles. Additionally, the AhR was seen in the oocyte nucleus in primordial, primary, and secondary follicles. AhR protein was not seen in other ovarian compartments such as the theca or interstitial cells. Dosing with VCD had no affect of AhR protein distribution or localization.

Since the results of the follicle counting experiment suggest an AhR-mediated effect and primordial and primary follicles were found to express the AhR, the effect of VCD on expression of AhR-mediated genes was examined. Using RT-PCR, mRNA encoding *glutathione-S-transferase* (GST) subunits *Ya1* and *Ya2* was measured in isolated follicles. Repeated dosing with VCD did not alter expression of *GST Ya1* or *Ya2* mRNA in fraction 1 or 2 follicles (Figure 2.5). Using western blotting, CYP 1A1 and 1A2 protein was examined in isolated follicles and liver. VCD dosing did not affect protein expression of either CYP 1A1 or 1A2 in liver or fraction 1 or 2 follicles (Figure 2.6).

VCD has previously been shown to cause loss of small pre-antral follicles in mice (Kao *et al.*, 1999). Using AhR-deficient mice (AhRKO), daily dosing with VCD caused a significant decrease in the number of small pre-antral follicles (primordial + primary; $p<0.05$; Figure 2.7). In heterozygous mice (+/-), treatment with VCD caused a non-significant trend to decrease small pre-antral follicles ($p=0.09$). As with rats, VCD did not alter large pre-antral follicle numbers in any genotype.

Figure 2.1: Effect of dosing with VCD and alpha-naphthoflavone (ANF) on rat pre-antral ovarian follicles. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), ANF (20 or 80 mg/kg, i.p.), or VCD and ANF. Primordial, primary, and secondary follicles were counted in every 40^{th} section as described in methods. Values are mean total follicles counted in each ovary ± standard error; means that share common superscripts are statistically similar ($p>0.05$). N=7-9.


Primordial Follicles
Treatment/Control Ratio
a
b
c
d
a, d
Primary Follicles
Treatment/Control Ratio
a
b
c
d
a, d
Secondary Follicles
Treatment/Control
a
a
a
a
a
Control
VCD
Hi ANF
Lo ANF & VCD
Hi ANF & VCD

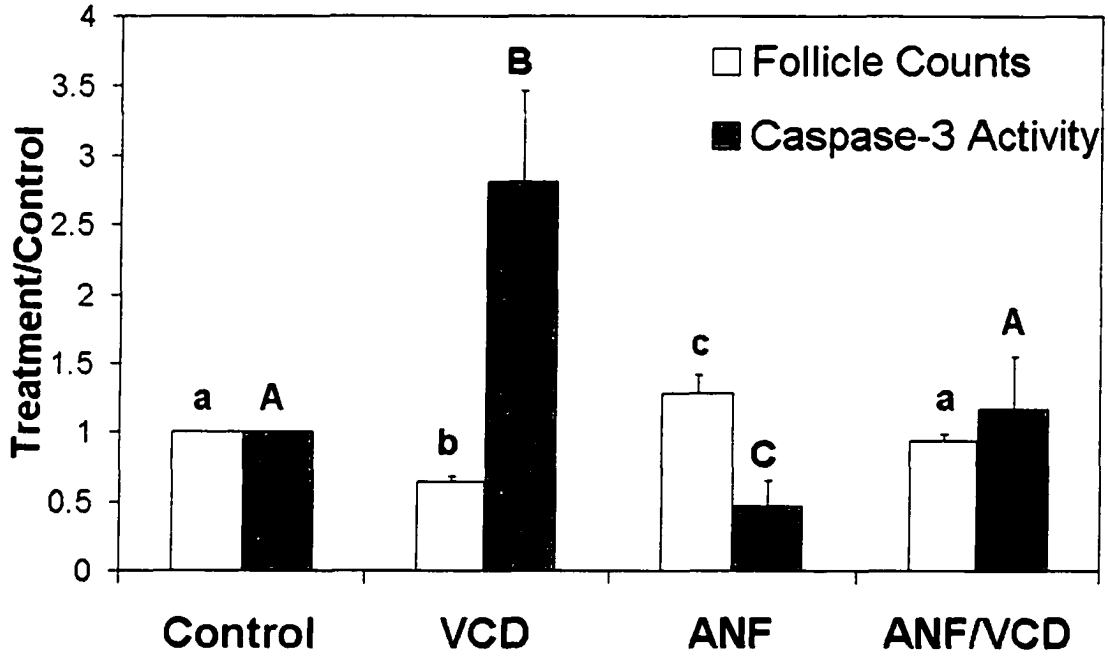


Figure 2.2: Effect of dosing with VCD and an AhR antagonist, alpha-naphthoflavone (ANF), on rat small pre-antral ovarian follicle numbers and caspase-3-like activity. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), ANF (80 mg/kg, i.p.) or VCD and ANF. Primary follicles were counted in every 40th section of ovaries as described in the methods. Caspase-3-like activity was measured in isolated fraction 1 follicles (25-100 μm) as described in the methods. Open bars; mean follicles counted in each ovary ± standard error (N ≥ 4). Shaded bars; mean fluorescence as a measure of caspase-3-like activity ± standard error (N=3); means that share common superscripts are statistically similar ($p>0.05$).

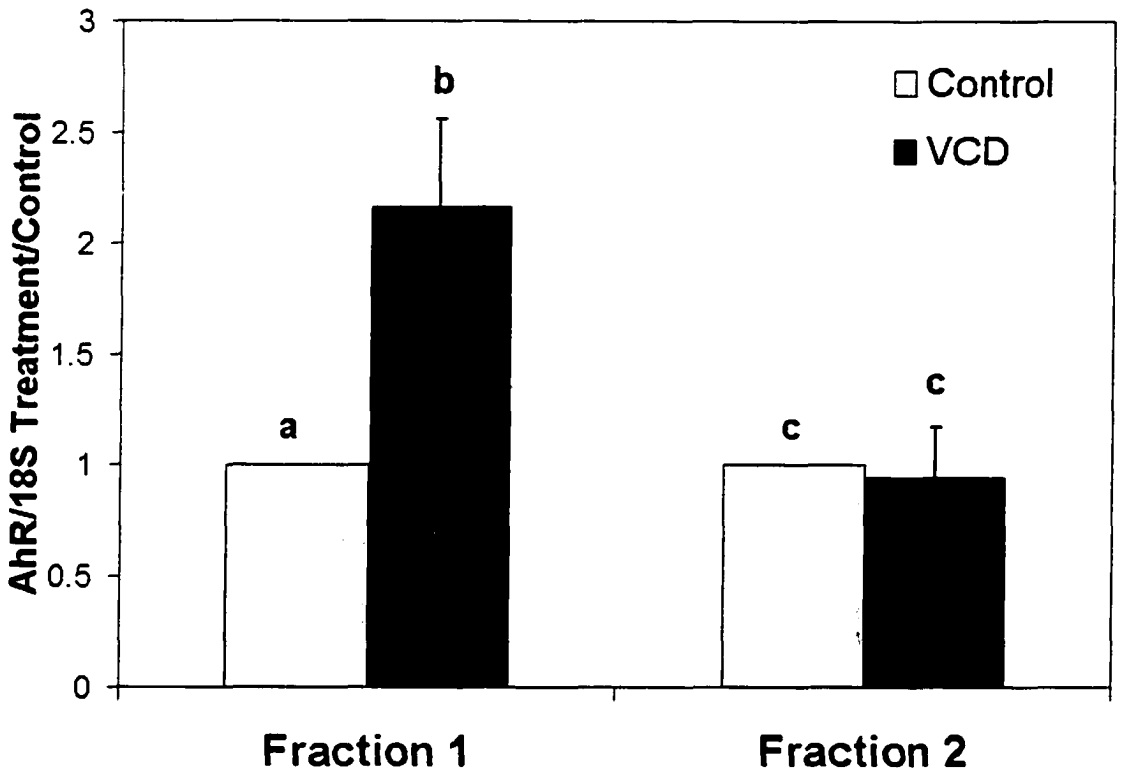
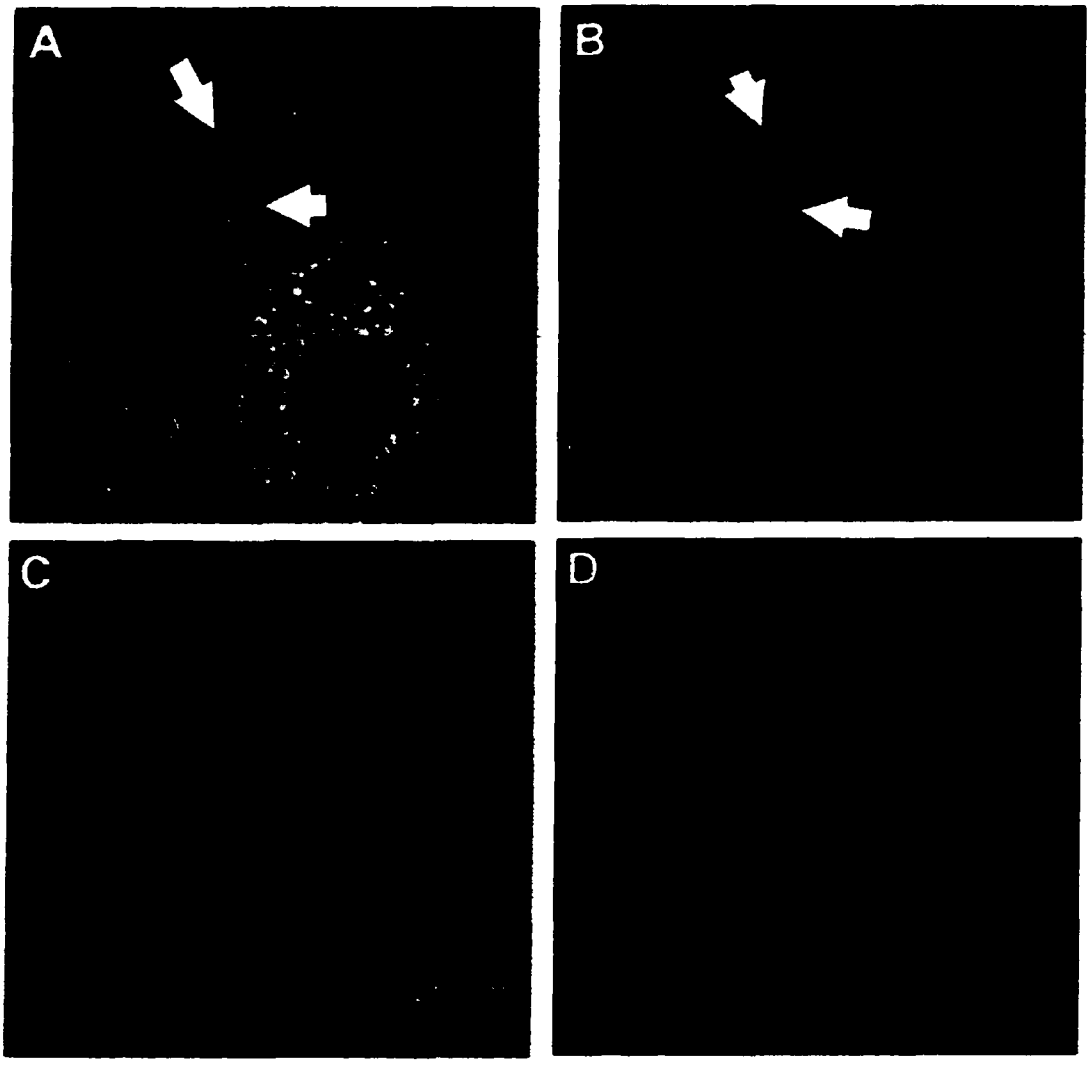


Figure 2.3: Effect of VCD dosing on *AhR* mRNA expression in fraction 1 and 2 ovarian follicles. F344 rats (d28) were dosed daily for 15 days with vehicle control or VCD (80 mg/kg, i.p.). Fraction 1 (25-100 μm) and fraction 2 (100-250 μm) follicles were isolated and analyzed by RT-PCR as described in methods. Values are means (±SE) of mRNA encoding *AhR*, normalized to *18S*, and expressed as VCD/control. (N=3-4); means that share common superscripts are statistically similar ($p>0.05$).

Figure 2.4: Distribution of AhR protein by confocal microscopy. Ovarian sections from d42 control rats were incubated with an anti-AhR antibody. Slides were analyzed on a Leica confocal microscope at 40X as described in the Methods. The green stain (YOYO-1) displays DNA in all cell nuclei; the red stain (Cy-5) represents anti-AhR antibody. Colocalization of the red and green stains appears yellow. Overlay of the two stains is seen in Box A, while the red stain only for Box A is seen in Box B. Boxes C and D represent the immunonegative slide, incubated with no primary antibody. Arrows highlight primordial follicles in Boxes A and B.


A
B
C
D

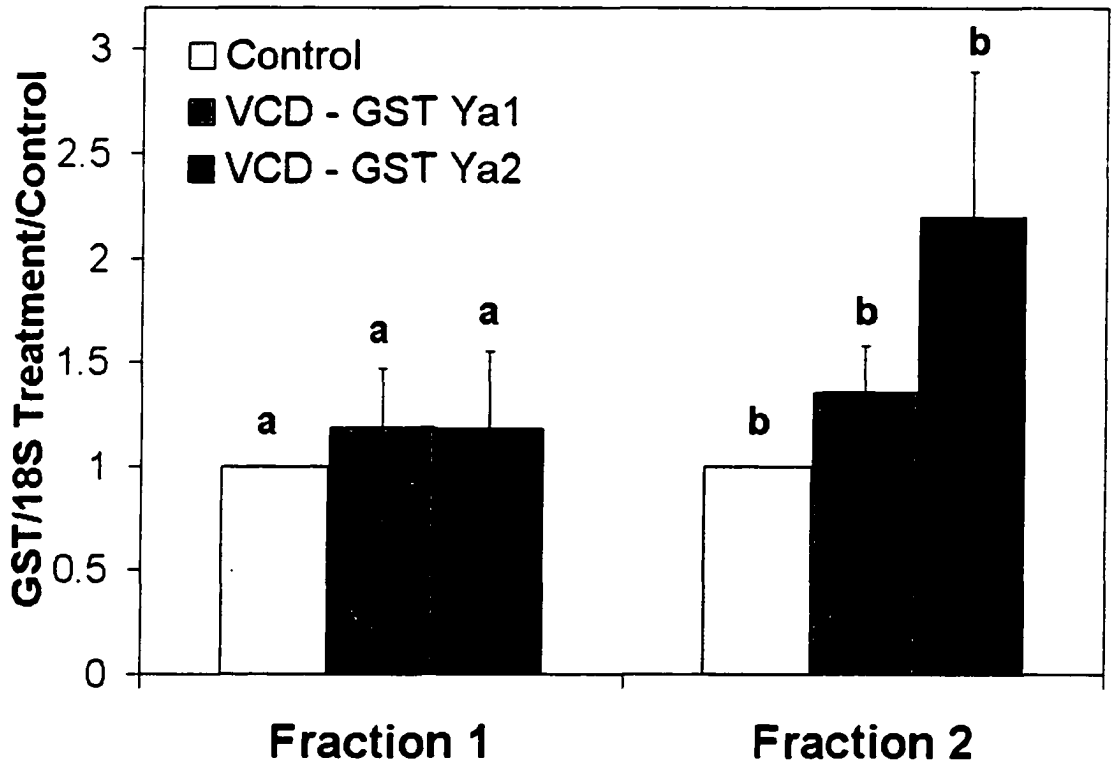


Figure 2.5: Effect of VCD on steady state levels of mRNA encoding two glutathione S-transferase subunits, *Ya1* and *Ya2*, in fraction 1 and 2 ovarian follicles. F344 rats (d28) were dosed daily for 15 days with vehicle control or VCD (80 mg/kg, i.p.). Fraction 1 (25-100 μm) and fraction 2 (100-250 μm) follicles were isolated and analyzed by RT-PCR. Values are mean (±SE) mRNA encoding *GST Ya1* or *Ya2*, normalized to *18S*. (N=3-4); significance was assigned at $p<0.05$. Means that share common superscripts are statistically similar ($p>0.05$).

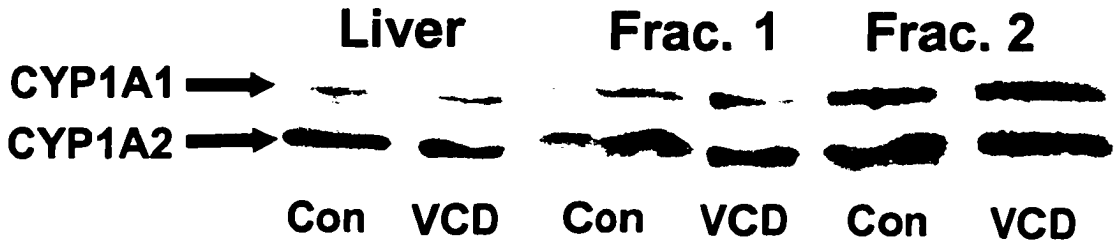


Figure 2.6: Representative western blot of CYP 1A1/1A2 in liver, fraction 1 follicles, and fraction 2 follicles. F344 rats were treated daily for 15 days with either vehicle control (sesame oil) or VCD (80 mg/kg). Ovaries and liver were removed and ovaries were dissociated for follicle isolation. 20 μg of protein from fraction 1 or 2 follicle or 10 μg of protein from liver cellular homogenate was separated by 12% SDS-PAGE and then transferred to nitrocellulose. CYP 1A1/1A2 protein was detected as described in the Methods.

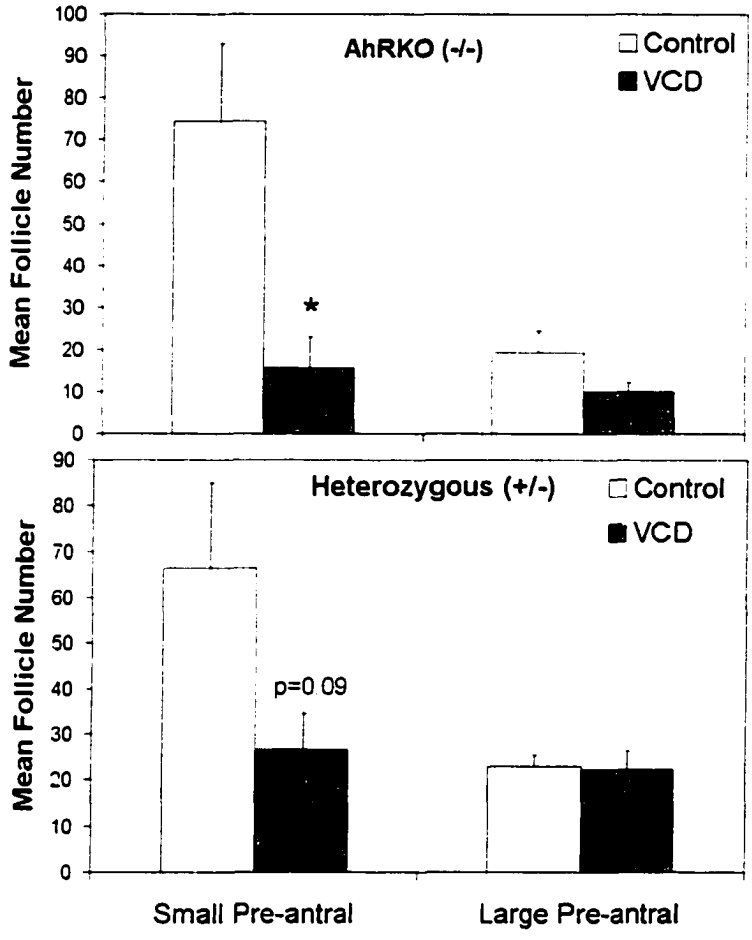


Figure 2.7: Effect of dosing with VCD on follicle number in AhR-deficient mice. AhR heterozygous (+/-) and AhR-deficient (-/-) mice were treated daily for 15 days with either vehicle control or VCD (80 mg/kg). Small pre-antral (primordial and primary; VCD-targeted) and large pre-antral (secondary; non-VCD-targeted) follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary ± standard error; (N=3-4); * indicates significant difference between control and treatment ($p<0.05$).

DISCUSSION

Co-treatment of rats with VCD and ANF, an AhR antagonist, prevented VCD-induced follicular toxicity in both primordial and primary follicles following 15 days of daily dosing. Therefore, to determine whether ANF and VCD alteration of follicle numbers reflect changes in the rate of atresia, an intracellular marker of apoptosis, caspase-3 activity, was measured in isolated small pre-antral follicles (VCD-targeted). Previous research has found that VCD-induced loss of primordial and primary follicles is accompanied by increased caspase-3-like activity specifically in isolated small pre-antral follicles (Hu *et al.*, 2001b). In the study reported here, treatment with ANF plus VCD prevented VCD-induced changes in both follicle number and caspase-3-like activity. These observations indicate that the protection from VCD-induced follicle toxicity resulting from inhibition of the AhR with ANF is via reduced apoptosis. Based on this study, it is unclear whether VCD and ANF are acting in the same pathway to modulate follicular loss by apoptosis or if these effects are a combination of two different points of regulation.

Interestingly, treatment with ANF alone resulted in a greater number of small follicles as compared to control. Inhibition of AhR activity caused by treatment with ANF alone also reduced caspase-3-like activity as compared to control. These results demonstrate that there is an inverse relationship between follicle number and caspase-3-like activity. Because mammals are born with a finite number of primordial follicles, this could not result from generation of more follicles in the ovary. Therefore, it is likely that inhibition of AhR activity by ANF dosing over the 15 day period decreases the normal

rate of atresia occurring in control animals. Alternatively, ANF could be diminishing the activation and recruitment of the smallest follicles to larger stages of development. Further studies are required to distinguish between these possibilities.

Alpha-naphthoflavone is known to modulate cytochrome P450 (CYP 450) expression and activity. ANF activates CYP 3A4 and inhibits CYP 1A1, 1A2, 2A6, 1B, 2B6, 2C8, and 2C9 expression and activity (Chang *et al.*, 1994; Maenpaa *et al.*, 1998). Rats in this study were dosed with the bioactive form of the toxicant, VCD, which does not require metabolism to cause ovotoxicity. Additionally, VCD is detoxified by microsomal epoxide hydrolase to an inactive tetrol (Flaws *et al.*, 1994b). Therefore, alterations in the CYP 450s caused by ANF should not have affected the metabolism of VCD (either bioactivation or detoxification), and the interactions of ANF and VCD on primordial and primary follicles are most likely a direct ovarian effect, rather than an indirect effect involving hepatic metabolism.

Because the protection against VCD-induced follicle loss afforded by ANF may be via inhibiton of an AhR receptor-mediated mechanism, an investigation of expression and distribution of the AhR in small pre-antral follicles was made. Expression of the AhR was observed in VCD-targeted small preantral follicles as demonstrated at the mRNA and protein level. Recent studies using genetically modified mice have suggested that the AhR may regulate atresia in mice and the absence of this receptor can decrease the normal rate of follicular attrition (Robles *et al.*, 2000). Additionally, VCD has been reported to enhance the normal rate of atresia (Borman *et al.*, 1999). Repeated dosing

with VCD increased expression of mRNA encoding the *AhR*. Therefore, one way VCD may be accelerating atresia is via upregulation of the *AhR* to increase atresia.

The regulation of expression of the *AhR* is not well understood. In humans, *AhR* mRNA is differentially regulated in tissues throughout the body in the absence of exogenous ligand by unknown mechanisms (Hahn, 1998). It has not been reported that the AhR promoter contains a xenobiotic response element (XRE) to regulate expression of its own receptor. However, AhR ligands have been identified that alter expression of mRNA encoding the *AhR*. Treatment of rats or cells with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) both increased and decreased expression of AhR mRNA and protein, depending on the dose and length of exposure (Franc *et al.*, 2001; Hahn, 1998; Pitt *et al.*, 2001). Therefore, it is unclear how the AhR is regulated both in the absence and presence of xenobiotics.

Activation of the AhR is known to regulate several genes and proteins (Denison and Heath-Pagliuso, 1998). Known ligands (i.e. PAHs) of the AhR cause increases in expression of CYP 1A1 and the GST subunits Ya1 and Ya2 (Lindros *et al.*, 1998; Paulson, 1989). VCD did not increase expression of mRNA encoding *GST Ya1* or *Ya2*, nor protein for CYP 1A1 in VCD-targeted fraction 1 follicles, non-VCD-targeted fraction 2 follicles, or liver. This provides evidence that VCD is not impacting that particular function of the AhR. However, it is thought that the AhR can also mediate its effects by non-genomic pathways (Gonzalez and Fernandez-Salguero, 1998; Gu *et al.*, 2000). The AhR has been demonstrated to interact with another transcription factor, the estrogen receptor, as well as the pro-apoptotic cytokine, transforming growth factor β (TGFβ;

Gonzalez and Fernandez-Salguero, 1998; Safe *et al.*, 1998). Therefore, VCD may interact with an AhR-mediated pathway via a non-genomic mechanism.

AhR protein demonstrated a unique staining pattern in VCD-targeted primordial and primary follicles. The AhR was localized in the granulosa cell nucleus in secondary and larger follicles (non-VCD targeted). The unique localization of the AhR in the nucleus (instead of the cytoplasm) of granulosa cells in large follicles has been previously demonstrated by Robles *et al.*, 2000. The nuclear compartmentalization provides further evidence that the AhR may be functioning in non-classical pathways in the ovary. In VCD-targeted primordial and primary follicles, AhR protein staining was also observed in the oocyte nucleus. This difference in distribution between follicle types may relate to the selectivity of VCD for primordial and primary follicles. If the AhR is involved with VCD-induced follicle loss, then the additional expression of the AhR in the vulnerable germ cells of the smallest follicles could explain why these follicles are suspectible to VCD.

In order to determine whether VCD-induced follicle loss occurs via alteration in AhR activity, the effect of VCD-dosing on follicle number in AhR-deficient mice was assessed. AhRKO mice are fertile, but they produce fewer total and live pups (Abbott *et al.*, 1999). Additionally, AhRKO mice have compromised immune systems, heart hypertrophy, dermal abnormalities, and reduced liver size (Gonzalez and Fernandez-Salguero, 1998). Since VCD only affects small pre-antral ovarian follicles, the non-ovarian AhRKO-associated phenotypes would not be predicted to interfere with VCD action in the ovary. The AhRKO (-/-) mice were susceptible to VCD-induced follicle

loss suggesting that VCD is accelerating atresia by an AhR-independent pathway. Atresia is likely controlled by many pathways. Because AhRKO mice are still subject to a degree of ovarian atresia, the AhR is probably only one of the components involved in this physiological process. Therefore, VCD may be interacting with an AhR pathway, but also working through other mechanisms to cause accelerated atresia. Alternatively, there could be a species difference between mice and rats in the pathway(s) by which VCD causes follicle loss. Many strains and species are known to have dramatic differences in their sensitivity to AhR ligands (Denison and Heath-Pagliuso, 1998). Subsequently, the AhR may be a more important component of VCD-induced follicle loss in rats, as compared with mice.

Taken together, the results presented here support that physiological inhibition of the AhR can protect against the normal rate of atresia in primordial and primary follicles, as well as VCD-induced follicle loss. However, this inhibition does not appear to result from a direct interaction between VCD and the AhR. Therefore, it is likely that acceleration of atresia by AhR and VCD in small pre-antral follicles occurs by parallel or redundant intracellular pathways. Understanding general mechanisms of toxicant-induced follicle loss as well as cell signaling in normal ovarian atresia can provide insight into factors that regulate the reproductive life-span in women.

CHAPTER 3

17β-ESTRADIOL AFFORDS PROTECTION AGAINST 4-VINYLCYCLOHEXENE DIEPOXIDE-INDUCED OVARIAN FOLLICLE LOSS IN FISCHER-344 RATS

ABSTRACT

Repeated dosing with 4-vinylcyclohexene diepoxide (VCD) accelerates atresia via apoptosis in primordial and primary follicles in ovaries of rats. The mechanisms which control atresia and VCD-induced toxicity are unknown; however, they could involve 17β-estradiol (E_2). Atresia slows as animals enter puberty while circulating E_2 levels increase with the onset of cyclicity. This inverse relationship suggests that E_2 may be involved in the control of atresia. Therefore, this study was designed to determine whether dosing of immature rats with E_2 could protect follicles normally destroyed by VCD-induced apoptosis. Female F344 rats were dosed daily with E_2, estrogen receptor (ER) analogs, and/or VCD for 15 days. VCD alone caused a 50% reduction in primordial and primary follicles. Co-injection with E_2 (0.1 mg/kg) and VCD (80 mg/kg) selectively protected primary follicles from VCD-induced follicle loss. This protection was mimicked by an ER agonist, genistein (0.1 mg/kg), and prevented by an ER antagonist, 4-hydroxytamoxifen (2 mg/kg). VCD dosing increased caspase-3-like activity while concurrent treatment with genistein and VCD restored caspase-3-like activity to control levels. VCD dosing had no effect on circulating E_2 levels, uterine weight, E_2 binding to the ER, nor could it directly displace E_2 from ERβ. These observations support that ER-

mediated protection against VCD-induced follicle toxicity is by a reduction of apoptosis in small pre-antral follicles, although VCD does not appear to directly interact with ER.

INTRODUCTION

Repeated daily dosing with the occupational chemical 4-vinylcyclohexene diepoxide (VCD) destroys primary and primordial (small pre-antral) follicles in the ovaries of rats and mice (Flaws *et al.*, 1994a; Smith *et al.*, 1990b). Mammals are born with a finite number of primordial follicles and the depletion of this follicle pool results in ovarian failure (menopause in humans). Therefore, any toxicant that targets these small follicles extensively can cause premature ovarian failure.

Previous studies have demonstrated that VCD causes follicle loss by apoptosis and accelerates the natural process of atresia (Borman *et al.*, 1999; Springer *et al.*, 1996c). However, the exact mechanisms of VCD-induced follicle toxicity are unknown. Although it is not thought to act as a direct endocrine disruptor, VCD has previously been found to reduce uterine weights, interrupt normal estrus cycles in adult rats, and eventually cause premature ovarian failure in rats and mice (Flaws *et al.*, 1994a; Hooser *et al.*, 1994; Mayer *et al.*, 2001). These effects are most likely the result of reduced follicular development as the pool of small follicles for recruitment is depleted. While a human exposure to VCD at concentrations found to cause toxicity in rodents is unlikely, VCD is used as a model compound. Because it is specific for small pre-antral follicles and alters a natural process in the ovary, VCD has been used to study follicle toxicants

which target small pre-antral follicles, as well as a tool to dissect the atretic signaling pathway.

The specific factors that control the determination between follicular survival versus death by atresia (apoptosis) are not well understood. Additionally, those factors which regulate follicular survival appear to differ between various stages of follicles. Many studies have examined the mechanism in large antral follicles. The primary site of induction of apoptosis in follicular atresia is thought to be within granulosa cells in larger follicles and is known to involve several factors (for extended description, see Chapter 1; Billig *et al.*, 1993). However, factors that regulate survival versus atresia in small pre-antral follicles are not as well known.

In the past, small pre-antral follicles were not shown to express estrogen receptor (ER) α (Iwai *et al.*, 1990). Thus, it was assumed for many years that the control of development in these follicles is independent of E_2. However, ERβ has recently been discovered and reported to be expressed in granulosa cells of small pre-ovulatory ovarian follicles (Ahluwalia *et al.*, 1998; Kuiper *et al.*, 1996). Thus, the possibility of a regulatory role in pre-antral follicle development seems more plausible. A role for 17β-estradiol (E_2) in controlling apoptosis has been well established in many tissues throughout the body. It was found to suppress apoptosis in the rabbit corpus luteum via an impact on expression of the *bcl-2* gene family (Goodman *et al.*, 1998). 17β-Estradiol has also been found to exert anti-apoptotic effects in endothelial cells (Spyridopoulos *et al.*, 1997), peripheral blood mononuclear cells (Evans *et al.*, 1997), neurons (Garcia-

Segura *et al.*, 1998), MCF-7 breast cancer cells (Wang and Phang, 1995), osteocytes (Tomkinson *et al.*, 1997), and cardiac myocytes (Pelzer *et al.*, 2000).

Ovarian atresia, which occurs via apoptosis, is a dynamic process. The rate of physiological atresia slows as a female enters puberty while circulating E_2 levels increase from non-detectable (<10 pg/ml in rats) to adult levels (30-60 pg/ml in rats) with the onset of estrous cycles (Uilenbroek *et al.*, 1975). This inverse relationship between the rate of atresia and circulating 17β-estradiol suggests a role for E_2 in the normal control of atresia. Exposure to VCD is known to accelerate atresia. Therefore, the following study was designed to test the hypothesis that VCD-targeted small pre-antral follicles in immature animals can be protected from VCD-induced apoptosis by concurrent dosing with E_2.

METHODS

See Appendix B.

RESULTS

Following 15 days of repeated dosing, circulating levels of E_2 were significantly higher ($p<0.0001$; mean $E_2 = 3.50 \pm 0.24$ ng/ml; Table 3.1) in rats treated with E_2 than in those not treated with E_2 (less than 23 pg/ml). Treatment with E_2 also increased uterine weight ($p<0.0001$) as compared to non-E_2 treated animals (Table 3.2). Treatment with

the ER analogs, genistein or 4-hydroxytamoxifen, or the follicle toxicant VCD had no effect on circulating E_2 levels (Table 3.1). However, treatment with the estrogen receptor antagonist, 4-hydroxytamoxifen, prevented E_2-induced uterine weight increases, resulting in uterine weights equivalent to vehicle treated control rats (Table 3.2).

Following 15 days of daily dosing, VCD induced a significant loss ($p<0.05$) of primordial and primary follicles compared to controls as previously described (Flaws *et al.*, 1994a). When rats were co-injected with VCD and E_2, there was a significant loss ($p<0.05$) of primordial follicles, however, primary follicle numbers were equivalent to controls (Figure 3.1). Because only primary follicles demonstrated this E_2-induced protective effect, the following results will only be reported for this follicle population. For primordial follicle results, see Appendix A.2, Figures A.5 and A.6.

Following 15 days of dosing with E_2 alone, there was a non-significant trend ($p=0.15$) for primary follicles to be increased as compared to control (Figure 3.2). Dosing with the estrogen receptor agonist genistein demonstrated a pattern of results similar to those seen with the endogenous ER ligand, E_2 ($p=0.09$; Figure 3.2). Compared with controls, concurrent dosing with genistein and VCD resulted in no difference in primary follicle numbers (Figure 3.2). Following concurrent dosing with VCD, E_2, and the estrogen receptor antagonist 4-hydroxytamoxifen (TAM), there was a significant loss of primary follicles as compared to control (Figure 3.3).

In order to examine the effect of the ER receptor analog genistein on apoptosis, caspase-3-like activity was measured in isolated pre-antral follicles. In fraction 1 follicles, repeated daily dosing with VCD caused an increase ($p<0.05$) in caspase-3

activity as compared to control (Figure 3.4). However, following treatment with either genistein or genistein and VCD, caspase-3-like activity levels were similar to control (Figure 3.4). There were no effects in fraction 2 follicles (secondary follicles) for any treatment (Appendix A.2, Figure A.9).

Because the results of dosing suggested an estrogen receptor-mediated effect, the expression and localization of the estrogen receptor isoforms alpha and beta (ERα and ERβ) were examined. Using RT-PCR, the presence of mRNA encoding *ERα* and *ERβ* was detected in isolated fractions of small preantral follicles (25-100 μm; Figure 3.5).

In order to verify protein expression for ERα and ERβ in VCD-targeted follicles, immunohistochemical staining and confocal microscopy were used. Consistent with the RT-PCR observations, ERβ protein was more widely distributed in rat ovaries as compared to ERα (Figures 3.6 A/B). When images were merged, colocalization of the red stain for either ERα or ERβ and the green nuclear stain YOYO-1 appeared yellow, therefore indicating that the protein of interest is located in the nucleus. ERα protein was localized to the oocyte nuclei of all sizes of follicles (Figure 3.6A), whereas ERβ protein staining was seen in the cytoplasm and nucleus of both oocytes and granulosa cells in primordial and small primary follicles (Figure 3.6B). However, in larger follicles, ERβ staining was compartmentalized within the nucleus of oocytes and granulosa cells. VCD had no effect on the distribution or localization of either ERα or ERβ as determined with densitometric analysis of staining intensity (data not shown). To verify the antibodies

used in this analysis, western blotting demonstrated a single band for both ERα and ERβ at the expected size (Figure 3.7).

As determined by a receptor assay, VCD dosing did not alter whole ovarian ER (α+β) receptor binding of E_2 (mean control $K_d = 2.94x10^{-10} \pm 0.32 x10^{-10}$; mean VCD $K_d = 3.76x10^{-10} \pm 0.34 x10^{-10}$) or total ovarian estrogen receptor number (mean control = $5.11x10^{-12} \pm 1.3x10^{-12}$ mole ER/mg protein; mean VCD = $4.78x10^{-12} \pm 0.67x10^{-12}$ mole ER/mg protein). In a competitive binding assay, VCD was not able to displace E_2 from ERβ at concentrations less than 0.1 M (Figure 3.8).

Table 3.1: Circulating estradiol levels in rats treated daily for 15 days in ER analog study (d28-42).

Treatment	Serum E_2 ± SE (pg/ml)
Control (d28)	0.22 ± 0.2
Control (d42)	4.0 ± 3.0
VCD	N.D.
Estradiol	3714 ± 714
Estradiol & VCD	3128 ± 172
Genistein	N.D.
Genistein & VCD	N.D.
Estradiol & 4-Hydroxytamoxifen	3517 ± 131
E_2, VCD, & 4-Hydroxytamoxifen	3887 ± 335

N.D. = Not detectable

Control = sesame oil, i.p.

VCD = 80 mg/kg, i.p.

Estradiol = 0.1 mg/kg, s.c.

Genistein = 0.1 mg/kg, i.p.

4-Hydroxytamoxifen = 2 mg/kg, i.p.

Table 3.2: Average uterine weight as a percent of body weight following dosing between d28-42 of age.

Treatment	% Uterine wt/body wt ± SE
Control, d42	0.109 ± 0.012
VCD	0.124 ± 0.019
Estradiol	0.306 ± 0.016*
E_2 & VCD	0.313 ± 0.040*
OH-Tamoxifen & E_2	0.148 ± 0.003
E_2, Tam, & VCD	0.150 ± 0.008

N = 4, * different from control, $p<0.0001$

VCD = 80 mg/kg, i.p.

17β-estradiol (E_2) = 0.1 mg/kg, s.c.

OH-Tamoxifen (Tam) = 2 mg/kg, i.p.

Table 3.3: Circulating estradiol levels in rats treated daily for 15 days in dose response study (d28-42).

Treatment	Serum E_2 ± SE (pg/ml)
Control	38 ± 0.2
VCD	27 ± 3.0
Low Estradiol	51 ± 3
Low Estradiol & VCD	89 ± 14
Mid Estradiol	119 ± 3
Mid Estradiol & VCD	95 ± 23
High Estradiol & VCD	983 ± 86

Control = sesame oil, i.p.

VCD = 80 mg/kg, i.p.

Low Estradiol = 0.001 mg/kg, s.c.

Mid Estradiol = 0.01 mg/kg, s.c.

High Estradiol = 0.1 mg/kg, s.c.

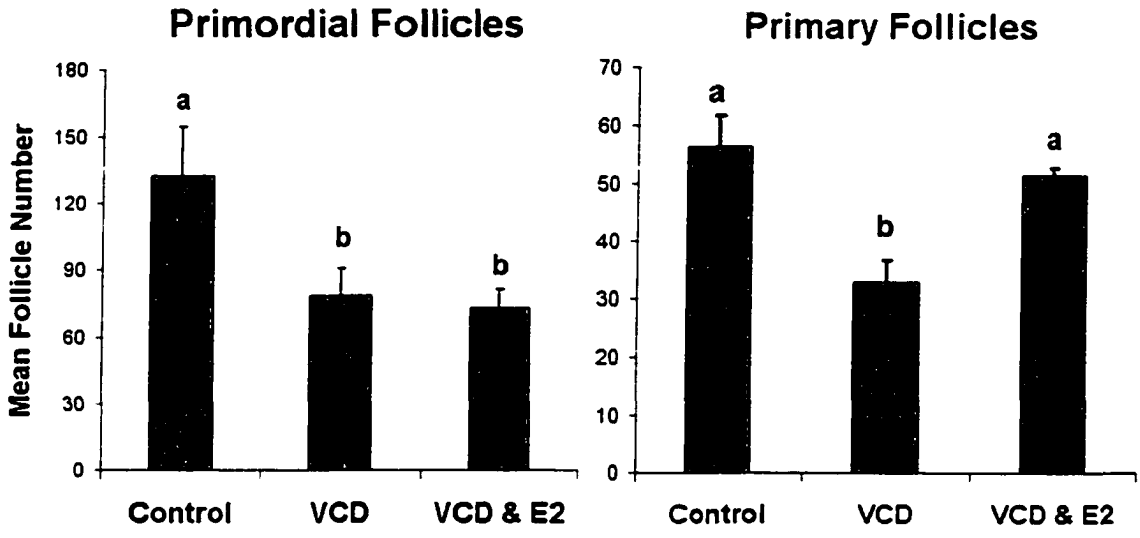


Figure 3.1: Effect of dosing with VCD and 17β-estradiol (E_2) on rat small pre-antral ovarian follicles. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.) or VCD and E_2 (0.1 mg/kg, s.c.). Primordial and primary follicles were counted in every 40^{th} section after histological staining. Values are mean total follicles counted in each ovary ± standard error; means that share common superscripts are statistically similar ($p>0.05$). $N \geq 10$ rats per treatment.

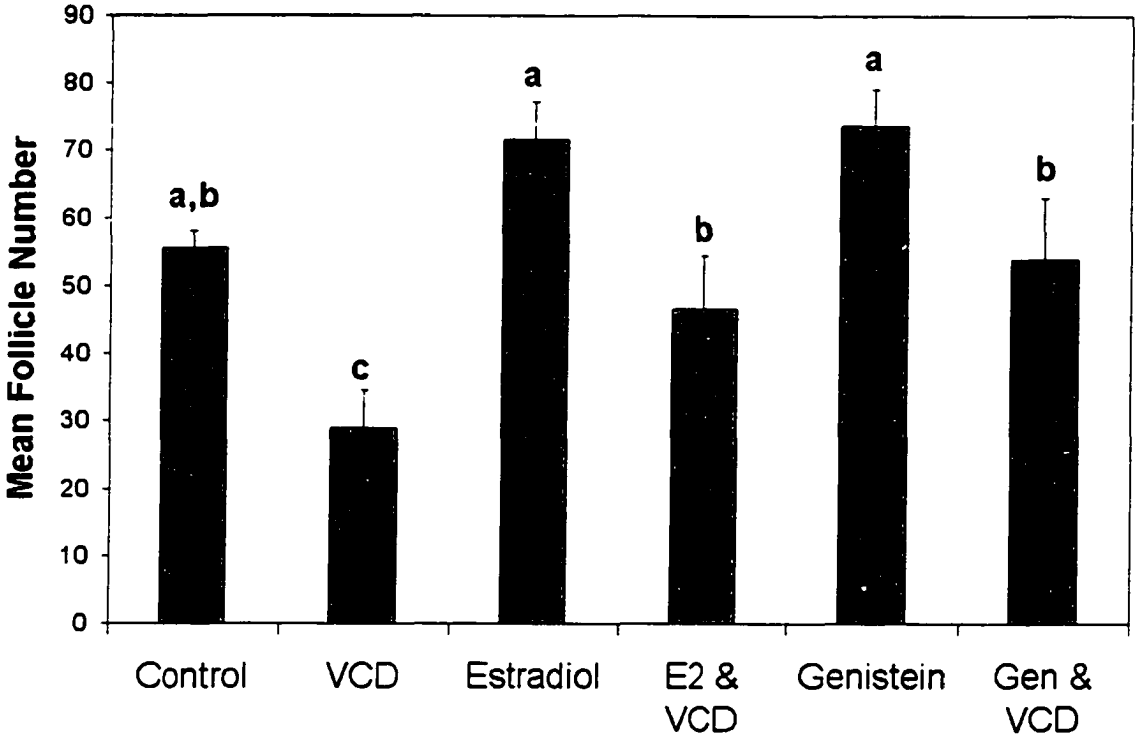


Figure 3.2: Effect of dosing with VCD, 17β-estradiol (E_2), and an ER agonist, genistein (Gen), on rat primary follicles. F344 rats (d28) were dosed daily for 15 days with one or more of the following treatments: vehicle control (sesame oil), VCD (80 mg/kg, i.p.), E_2 (0.1 mg/kg, s.c.), or genistein (0.1 mg/kg, i.p.). Primary follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary ± standard error; means that share common superscripts are statistically similar ($p>0.05$). $N \geq 4$ rats per treatment.

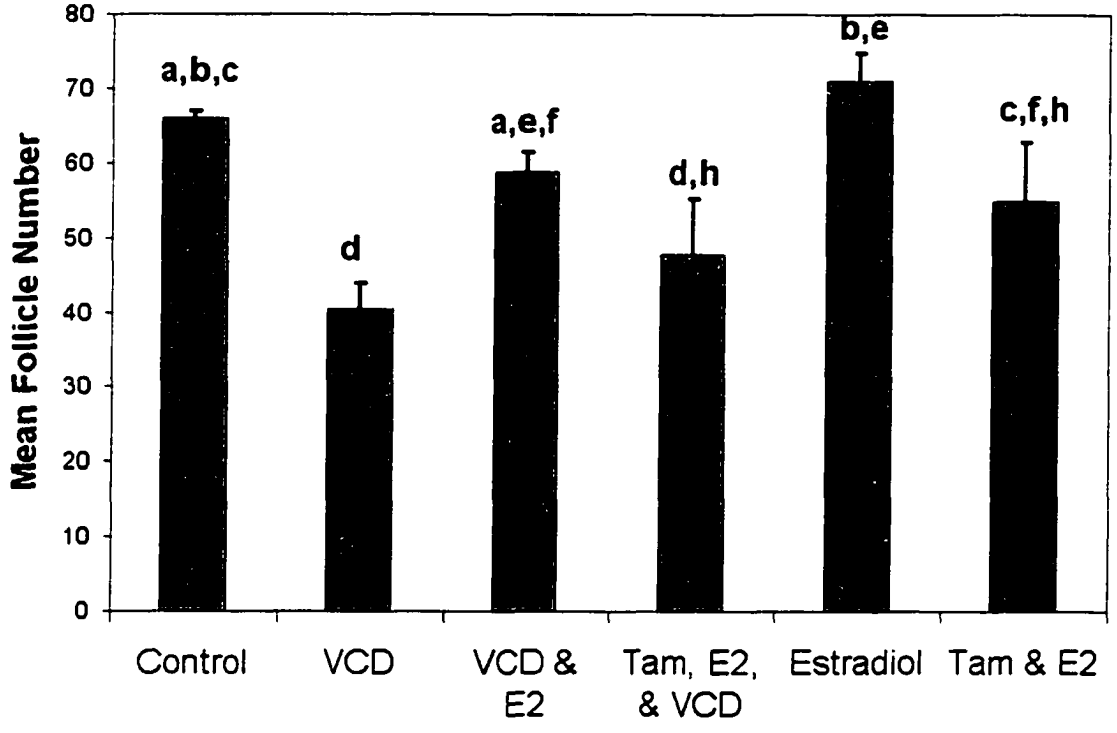


Figure 3.3: Effect of dosing with VCD, 17β-estradiol (E_2), and an ER antagonist, 4-hydroxytamoxifen (Tam), on rat primary follicles. F344 rats (d28) were dosed daily for 15 days with vehicle control (sesame oil), VCD (80 mg/kg, i.p.) ± E_2 (0.1 mg/kg, s.c.), or 4-hydroxytamoxifen (Tam, 2 mg/kg, i.p.), VCD, and E_2. Primary follicles were counted in every 40^{th} section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means that share common superscripts are statistically similar ($p>0.05$). $N \geq 4$ rats per treatment.

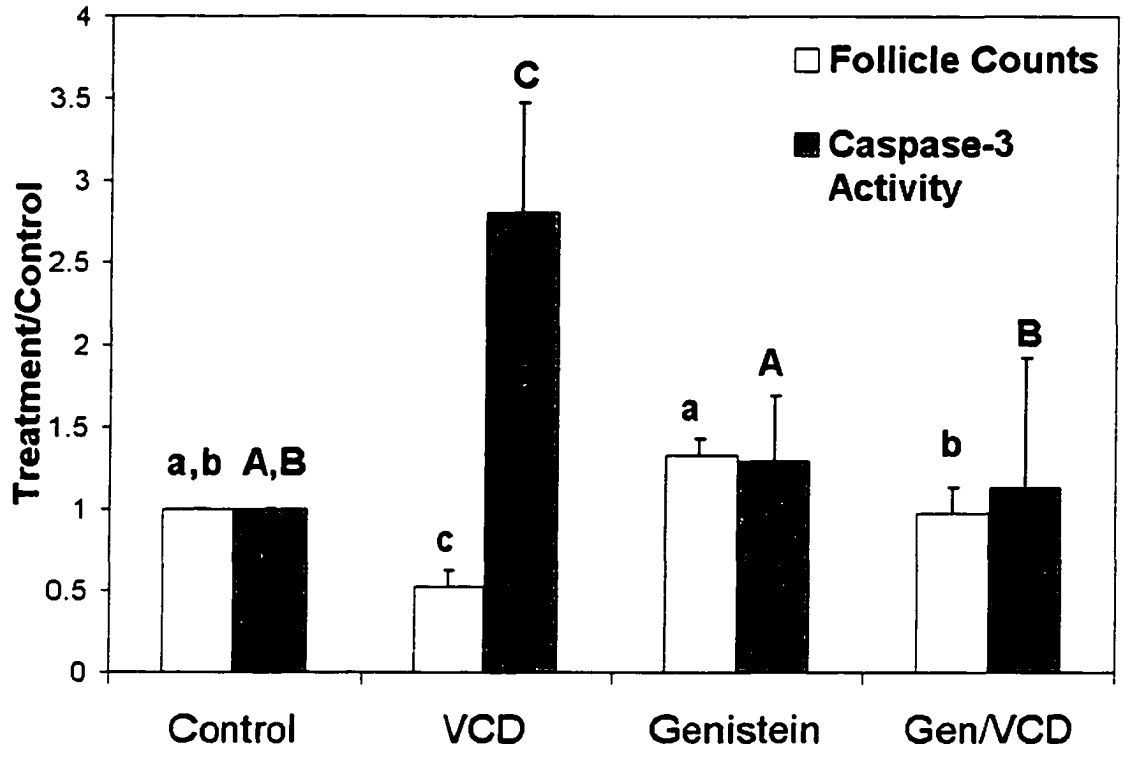


Figure 3.4: Effect of dosing with VCD and an ER agonist, genistein (Gen), on rat small pre-antral ovarian follicle numbers and caspase-3-like activity. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), genistein (0.1 mg/kg, i.p.) or VCD and genistein. Primary follicles were counted in every 40th section of ovaries as described in materials and methods. Caspase-3-like activity was measured in isolated fraction 1 follicles (25-100 μm) as described in the materials and methods. Open bars; mean follicles counted in each ovary ± standard error (N ≥ 4 rats per treatment). Shaded bars; mean fluorescence as a measure of caspase-3-like activity ± standard error (N=3); means that share common superscripts are statistically similar ($p>0.05$).

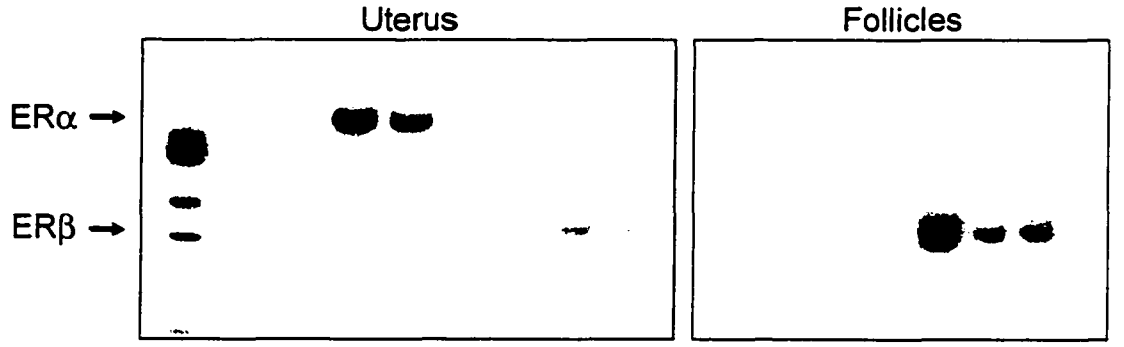


Figure 3.5: RT-PCR of estrogen receptor (ER) α and β in fraction 1 ovarian follicles (25-100 μm). Ovaries were collected from 3 groups of d42 control rats and dissociated for hand sorting of follicles. Each lane represents pooled follicles from 6 rats. ERα and ERβ were amplified by RT-PCR and visualized with autoradiography as described in Methods.

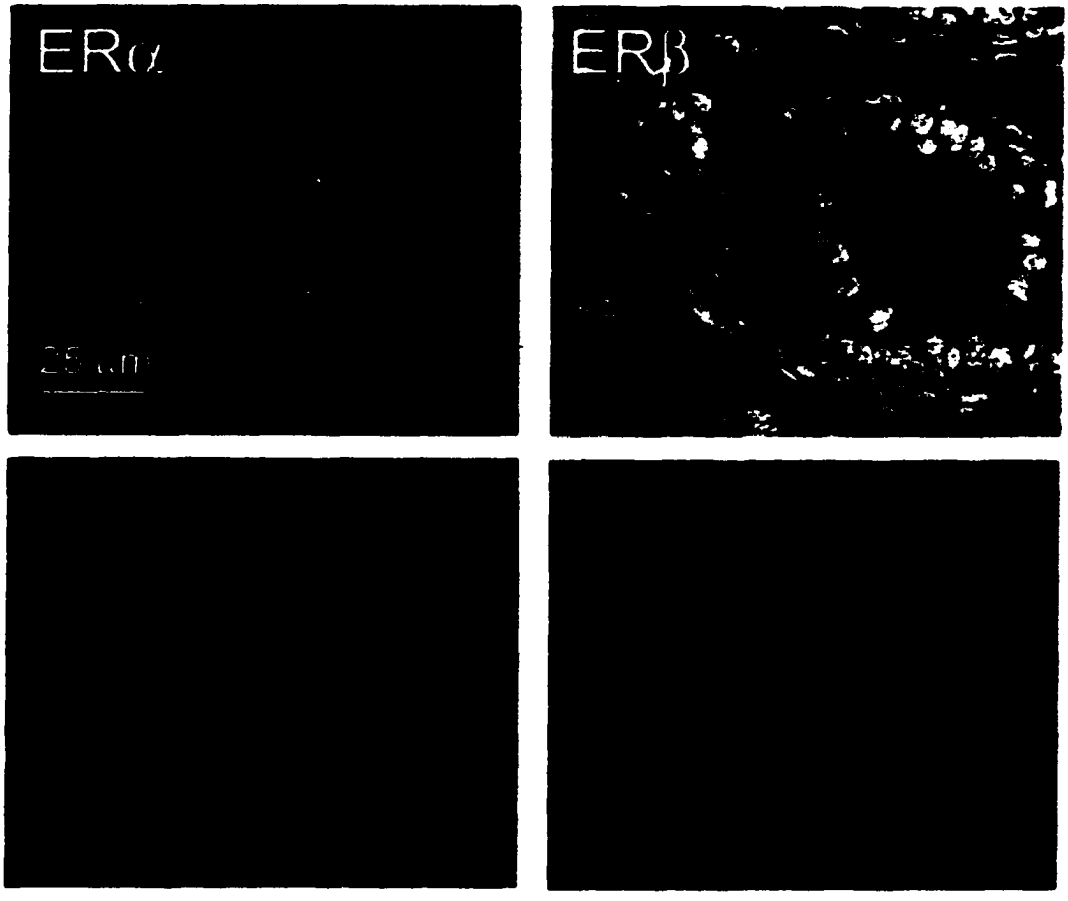


Figure 3.6: Distribution of ERα and ERβ protein by confocal microscopy. Ovarian sections from d42 control rats were incubated with either an anti-ERα or ERβ antibody. Slides were analyzed on a Leica confocal microscope at 40X as described in the Methods. The green stain (YOYO-1) displays DNA in all cell nuclei; the red stain (Cy-5) represents ERα (Box A) or ERβ (Box B) protein. Colocalization of the red and green stains appears yellow. Box C and D represent the immunonegative slide, incubated with no primary antibody.

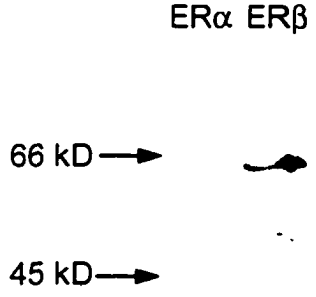


Figure 3.7: Representative western blot of ERα and ERβ in fraction 1 follicles. 50 μg of control fraction 1 follicle cellular homogenate was separated by 10% SDS-PAGE and then transferred to nitrocellulose. ERα and ERβ protein was detected as described in the Methods. Each antibody recognized a single band at the expected size (ERα = 66 kD, ERβ = 65 kD).

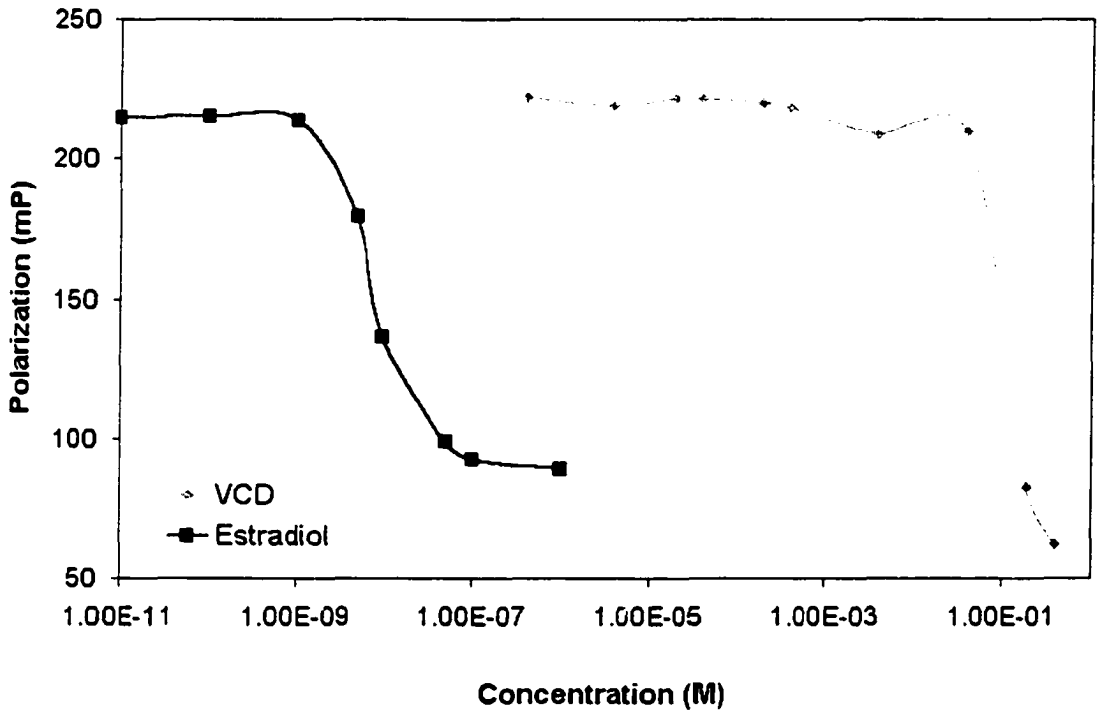


Figure 3.8: ERβ competition curve. The ability of VCD to displace an estrogen analog from ERβ was measured using a fluorescent estrogen (Fluormone™ ES2). Various concentrations of VCD were added and the shift in polarization as the fluorescent estrogen was displaced from the receptor was measured with the Beacon 2000 Fluorescence Polarization Instrument.

DISCUSSION

17β-Estradiol has been found to be protective against apoptosis in several tissues and cell types. The mechanism of protection is unclear and appears to involve several pathways. Estradiol can work as a direct antioxidant since it has a phenolic group and can scavenge reactive oxygen species (Nathan and Chaudhuri, 1998). This is thought to contribute to the protective effects of E_2 in the brain and cardiovascular system. In MCF-7 cells, the response to E_2 appeared to change as the E_2 exposure continued. Initially, E_2 caused a non-specific protection of all phosopholipids from oxidation by the diazo radical initiator, AMVN (Schor *et al.*, 1999). Therefore E_2 was acting as a direct antioxidant. Following long term treatment with E_2, the expression of the cell survival gene *bcl-2* was increased, and only phosphatidylserine, a critical component in the apoptosis pathway, was protected from oxidation (Schor *et al.*, 1999). At this point, E_2 was acting as an indirect antioxidant acting via its receptor, causing alterations in gene regulation. E_2 has been demonstrated to prevent apoptosis via modulation of gene expression by several other methods as well including terminal dUTP nick-end labeling (TUNEL), dye exclusion, reduced caspase-3 activity, and reduced activity of two NFκB transcription factors (p65/RelA and p50; Pelzer *et al.*, 2000).

Previous studies in our laboratory indicated that VCD does not reduce glutathione levels or increase lipid peroxidation as measured by the incidence of TBARS (thiobarbituric acid reactive substances) in whole ovaries (Devine *et al.*, 2001). However, Springer *et al.* (1996a) demonstrated that repeated dosing with VCD (10 days)

induces expression of mRNA encoding the oxidative stress response gene *manganese superoxide dismutase* (*MnSOD*) in isolated small follicles (targeted by VCD). Upregulation of mRNA encoding *MnSOD* has been shown to be a general defense against multiple cellular stresses (Jeong *et al.*, 2001; Rohrhanz *et al.*, 2001). Therefore, VCD may damage follicles by GSH-independent pathways of oxidative stress leading to apoptosis.

Based on follicle counting in the present study, E_2 prevented or reversed VCD-induced follicle toxicity/apoptosis specifically in primary follicles. The effect was mimicked by an ER agonist, genistein. Using the ER antagonist, 4-hydroxytamoxifen, E_2-induced protection against follicle toxicity was prevented. This finding provides support that E_2 likely prevents follicle toxicity via a receptor-mediated pathway.

Previous research has found that VCD destroys primordial and primary follicles and enhances caspase-3-like activity specifically in isolated small pre-antral follicles (Hu *et al.*, 2001b). In the study reported here, treatment with genistein or genistein plus VCD had no effect on follicle number or capsase-3-like activity. The caspase-3-like activities demonstrate that there is a significantly greater amount of atretic follicles in the VCD-treated group, as compared with controls or genistein-treated rats. Therefore, genistein has no effect on follicle number or apoptosis by itself, however it is able to prevent VCD-induced changes in follicle number and caspase-3-like activity. These observations suggest that the ER-mediated protection from VCD-induced follicle toxicity is via reduced apoptosis.

The interactions of estradiol and VCD on primary follicles are most likely a direct ovarian effect, rather than an indirect effect involving hepatic metabolism. Previous studies have not found VCD-induced liver damage. VCD dosing does not alter liver weights or enzyme activity of either AST (aspartate aminotransferase) or ALT (alanine aminotransferase; unpublished observation). Further, there are no signs of histopathology in VCD-treated liver tissues (unpublished observation). Additionally, VCD was not found to change circulating E_2 levels; therefore it seems unlikely that dosing with VCD is altering E_2 metabolism.

Because the protective effect of E_2 against follicle toxicity may be via a receptor-mediated mechanism, demonstrating expression of ER receptor protein in small pre-antral follicles is important. Expression of both ERα and ERβ was observed in VCD-targeted small preantral follicles as demonstrated at the mRNA and protein level. ERβ appears to be more highly expressed than ERα in primordial and primary follicles. Additionally, relative to larger, more highly developed follicles, ERβ displayed a unique pattern of distribution in these small follicles as visualized by confocal microscopy. The ER is generally considered to be a nuclear protein. However, ERβ was found in the cytoplasm of both the oocyte and granulosa cells in primordial and primary follicles, whereas, it was only localized within the nuclear compartment in secondary and antral follicles. Since ERβ staining was seen in the cytoplasm, this indicates that ERβ could be exerting non-genomic effects. The specialized localization of this receptor could suggest that there may be a different role for ERβ in early stage pre-antral follicles as compared to larger pre-antral and antral follicles.

17β-Estradiol prevented VCD-induced follicle loss specifically in primary and not primordial follicles, which are also targeted by VCD. This is an interesting finding because both follicle types express ER α and β. Thus, VCD may be activating the recruitment of primordial follicles into the growing stages rather than killing them directly. Alternatively, while primordial follicles express the estrogen receptor, the receptor may be a nonfunctional protein at this stage of development.

Based on structure alone, VCD would not be predicted to bind ER. However, a variety of chemicals with non-predicted structures have been found to interact with the ER (Ekena *et al.*, 1997). Therefore, the ability of VCD to directly interact with ERβ was investigated. VCD was not able to displace E_2 from ERβ at concentrations below 0.1 M. This provides strong evidence that VCD does not compete directly for binding to the ER.

The protective effect against VCD-induced follicle destruction required pharmacological doses of E_2 (367 nmol/kg BW), whereas lower doses were ineffective (100 fold, Appendix A.2, Figure A.4). There are other reports in which E_2 has been shown to only exert protective effects at non-physiological concentrations. When pig luteal and follicular tissues were placed in culture with hydrogen peroxide, E_2 was able to prevent DNA fragmentation, but only at E_2 concentrations greater than 40 pg/ml (Murdoch, 1998). Pharmacologic doses (≥0.2 mg/kg) of E_2 were required to reduce plasma cholesterol levels and increase expression of *LDL receptor* mRNA in rats (Parini *et al.*, 2000). In another study, pretreatment with low doses of E_2 (2.28 μg/day) did not prevent death of rats due to ventricular fibrillation that was seen with high levels of ethinyl estradiol (30-60 μg/day; Healy *et al.*, 1991). The severity of collagen-induced

arthritis can be reduced by treatment with high levels of estradiol benzoate (≥0.2 mg/kg), however at estradiol benzoate treatment levels of 0.05 mg/kg or lower, these therapeutic effects were lost (Waksman *et al.*, 1996). Furthermore, suppression of symptoms associated with autoimmune disorders requires pharmacologic doses of E_2 and it is thought that this occurs by an anti-inflammatory mechanism (Okayasu *et al.*, 1981; Strigard *et al.*, 1990; Carlsten *et al.*, 1992; Carlsten and Tarkowsky, 1993; Ratkay *et al.*, 1994). Since the protective effect demonstrated here required pharmacological doses of E_2, it may be working through non-typical pathways involving novel receptor isoforms.

In whole ovary homogenates, cytosolic ER binding of 17β-estradiol was unchanged following VCD dosing. Likewise, using confocal microscopy, there was no change in staining intensity or distribution of ERα or ERβ protein following VCD dosing as compared to controls. Taken together, these findings support that VCD is not directly affecting ER expression and binding.

Collectively, the results presented here support that 17β-estradiol can protect against VCD-induced follicle loss. Additionally, this does not appear to result from a direct interaction between VCD and ER. This effect will be further investigated in order to elucidate the specific mechanism(s) of this protection. Understanding general mechanisms of toxicant-induced follicle loss as well as cell signaling in normal ovarian atresia can provide insight into factors that regulate the reproductive life-span in women.

CHAPTER 4

A POTENTIAL ROLE FOR HEAT SHOCK PROTEIN 90 IN VCD-INDUCED FOLLICLE LOSS

ABSTRACT

Repeated exposure to the industrial chemical 4-vinylcyclohexene diepoxide (VCD) induces ovarian primordial and primary follicle loss in rats and mice. Previous studies have suggested that both the aryl hydrocarbon receptor (AhR) and estrogen receptor (ERα and/or ERβ) are involved with this toxicity, however the specific role of these proteins is unknown. Heat shock protein 90 (HSP90) has been demonstrated to affect the signaling for both the AhR and ERα/β and also has been found to be a mediator of the signaling between these two receptors. Therefore, this study tested the hypothesis that HSP90 is involved with VCD-induced follicle loss. F344 rats (d28) were dosed daily for 15 days with either vehicle control (DMSO, i.p.), VCD (80 mg/kg, i.p.), or the HSP90 inhibitor geldanamycin (0.3-60 μg/kg, i.p.). Four hours after the final dose, ovaries were removed, fixed, sectioned, and stained for oocyte counting or for confocal microscopy using a polyclonal anti-HSP90 antibody. Using confocal microscopy, over 300 follicles were visualized and HSP90 staining was quantified using arbitrary densitrometric analysis. HSP90 staining was compartmentalized to the oocyte in pre-antral follicles in all stages of development. In VCD-targeted small primary follicles, cytosolic HSP90 protein in the oocyte was significantly decreased in VCD-treated animals as compared to controls ($p<0.05$). Some oocytes from VCD-treated rats completely lacked HSP90

protein staining. Treatment of rats with geldanamycin did not affect primary and secondary follicle numbers. Low doses of geldanamycin did cause of loss of primordial follicles, however higher doses had no effect. Combined treatment with VCD and geldanamycin showed no difference in follicle loss as compared to dosing with VCD alone. Dosing with VCD or analogs of the ER and AhR did not alter expression of HSP90 protein in small pre-antral follicles (fraction 1) as measured by western blotting. Therefore, HSP90 protein is decreased specifically in small primary follicles following VCD dosing, however, HSP90 is not likely to be an essential player in VCD-induced ovotoxicity.

INTRODUCTION

4-Vinylcyclohexene (VCH) is released during the manufacture of rubber tires, plasticizers, and pesticides (IARC, 1994a). Its toxic metabolite, 4-vinylcyclohexene diepoxide (VCD), selectively destroys primordial and primary follicles in the ovaries of rats and mice (Smith *et al.*, 1994b). Repeated dosing with VCD appears to destroy follicles by accelerating the basal rate of atresia, the endogenous process by which 99% of follicles die via apoptosis (Borman *et al.*, 1999). The exact pathway of VCD-induced acceleration of atresia is unknown, however, it is known to enhance several downstream events associated with apoptosis including *bax*, Caspase-3, and Cytochrome *C* (Borman *et al.*, 1999; Springer *et al.*, 1996b; Hu *et al.*, 2001a). The early upstream intracellular events in VCD-induced ovotoxicity have not been identified, however they appear to

involve both estrogen receptor (ER) and aryl hydrocarbon receptor (AhR) pathways (Chapters 2 and 3). The ER and AhR are transcription factors, both chaperoned by heat shock protein 90 (HSP90), that are known to interact with one another by many proposed mechanisms.

Heat shock protein 90 is a constitutively expressed member of the molecular chaperone family. HSP90 is usually found in the cytosol as a homodimer and is induced by such stresses as increased temperature, metabolic poisons, alcohols, and toxins (Ylikomi *et al.*, 1998). Following a stressful condition, HSP90 is upregulated ten-fold and this is thought to occur due to the presence of non-native or abnormally folded proteins (Buchner, 1999). HSP90 and other members of the chaperone family have been termed "chaparones" due to their ability to stabilize the native structure of other proteins by binding them and releasing them in the proper configuration. One of the most well characterized functions of HSP90 is as a protein chaperone in the steroid receptor family. HSP90 binds as a dimer to unliganded AhR and ER (Caruso *et al.*, 1999; Caplan, 1999; Ylikomi, 1998). The HSP90-binding and ligand-binding domains colocalize in both receptor proteins which suggests that HSP90 is displaced by ligand binding to the receptor (Chambraud *et al.*, 1990; Coumailleau *et al.*, 1995). HSP90 binding to the receptors prevents receptor binding to DNA in the absence of ligand either by steric hindrance or interference with receptor dimerization (Caruso *et al.*, 1999).

Since HSP90 is used in the signaling cascade of both ER and AhR, receptor interactions between ER and AhR could be mediated by heat shock protein 90 (HSP90). It is thought that HSP90 plays different roles in the transactivation properties of ER and

AhR. Using ER and AhR positive cells, HSP90 overexpression had no effect on ER-mediated transactivation, but reduced TCDD-induced AhR reporter plasmid expression by 60-70% (Caruso *et al.*, 1999). This suggests that AhR-HSP90 interactions are more stable than ER-HSP90 associations and therefore increased HSP90 expression can decrease AhR signaling, and not affect ER signaling. In cell culture, Ah-responsiveness is related to its ER content such that cell lines which do not express the ER (MDA-MB-231 and Andriamycin-resistant MCF-7) are non-responsive to AhR ligands. Conversely, ER-positive cell lines (ER transfected MDA-MB-231 and wild type MCF-7) are able to activate AhR-mediated transcription in response to AhR ligands (Vickers *et al.*, 1989; Cowan *at el.*, 1986; Thomsen *et al.*, 1994). This suggests that the presence of the ER may sequester HSP90, allowing AhR signaling to occur more readily. Immunohistochemistry has shown that the presence of the ER alters cellular HSP90 distribution. In ER-negative cell lines, HSP90 is a cytoplasmic protein. In ER-positive cell lines, HSP90 is found equally distributed between the nucleus and cytoplasm (Caruso *et al.*, 1999). This effect on HSP90 distribution could play a role in ER/AhR "cross-talk" by altering AhR transactivation.

HSP90 has been demonstrated to play a role in nuclear receptor signaling and apoptosis. VCD has also been shown to alter apoptosis and modulate nuclear receptor signaling pathways. Therefore this study was designed to look for potential roles of HSP90 in VCD-induced ovotoxicity.

METHODS

See Appendix B.

RESULTS

HSP90 protein was expressed in the nucleus and cytoplasm of oocytes in all sizes of follicles (Figure 4.1). Repeated dosing with VCD caused a loss of staining selectively in small primary follicles (Figure 4.2). When this staining was quantified using densitometric analysis, VCD was shown to reduce the cytoplasmic staining of the oocyte as compared to control ($p<0.05$; Figure 4.3). Dosing with VCD did not affect HSP90 staining intensity in primordial or large primary follicles (Figure 4.3) or secondary follicles (Appendix A.3, Figure A.11).

Geldanamycin binds HSP90 and blocks HSP90 ATPase activity, which is required for its *in vivo* function (Roe *et al.*, 1999). Repeated dosing with a HSP90 function inhibitor, geldanamycin, decreased primordial follicle number as well as decreased body weight when rats were treated with 0.3 or 1.2 μg/kg ($p<0.05$; Figures 4.4 and 4.5). However, higher doses did not change primordial follicle counts as compared to control. Primary and secondary follicle numbers were not affected at any dose of geldanamycin (Figure 4.5 and Appendix A.3, Figure A.12). Dosing with geldanamycin had no effect on VCD-induced ovotoxicity in any follicle size (Figure 4.6 and Appendix A.3, Figure A.13).

Expression of HSP90 protein was detected in VCD-targeted fraction 1 follicles by western blotting (Figure 4.7). Expression of HSP90 was also seen in liver and non-VCD-targeted fraction 3 follicles (Figure 4.7). Western blotting demonstrated a single band of the predicted molecular weight (90 kD) in all tissues examined. Dosing with VCD had no effect on HSP90 protein expression in fraction 1 or 3 follicles. Additionally, dosing with the ER agonist genistein, VCD and genistein, the AhR antagonist alpha-naphthoflavone, or ANF and VCD did not change HSP90 expression in fraction 1 follicles (Figure 4.7).

Figure 4.1: Distribution of Heat Shock Protein 90 (Hsp90) in rat ovary. D42 rat ovaries were prepared and stained with a Hsp90 antibody. Boxes A, C, E, and G show the overlay of Hsp90 staining (Cy-5; RED) and nuclear DNA (YOYO-1; GREEN) while Boxes B, D, F, and H show the Cy-5 stain only. Hsp90 is seen in the cytoplasm and nucleus of oocytes in primordial, primary, and secondary follicles as indicated by arrows (A, B) and antral follicles (C, D). Cytoplasmic staining of the oviduct (indicated by arrow) was of similar intensity of that seen in the oocyte of follicles (E, F). An immunonegative slide incubated in the absence of primary antibody demonstrated no Hsp90 staining (G, H).

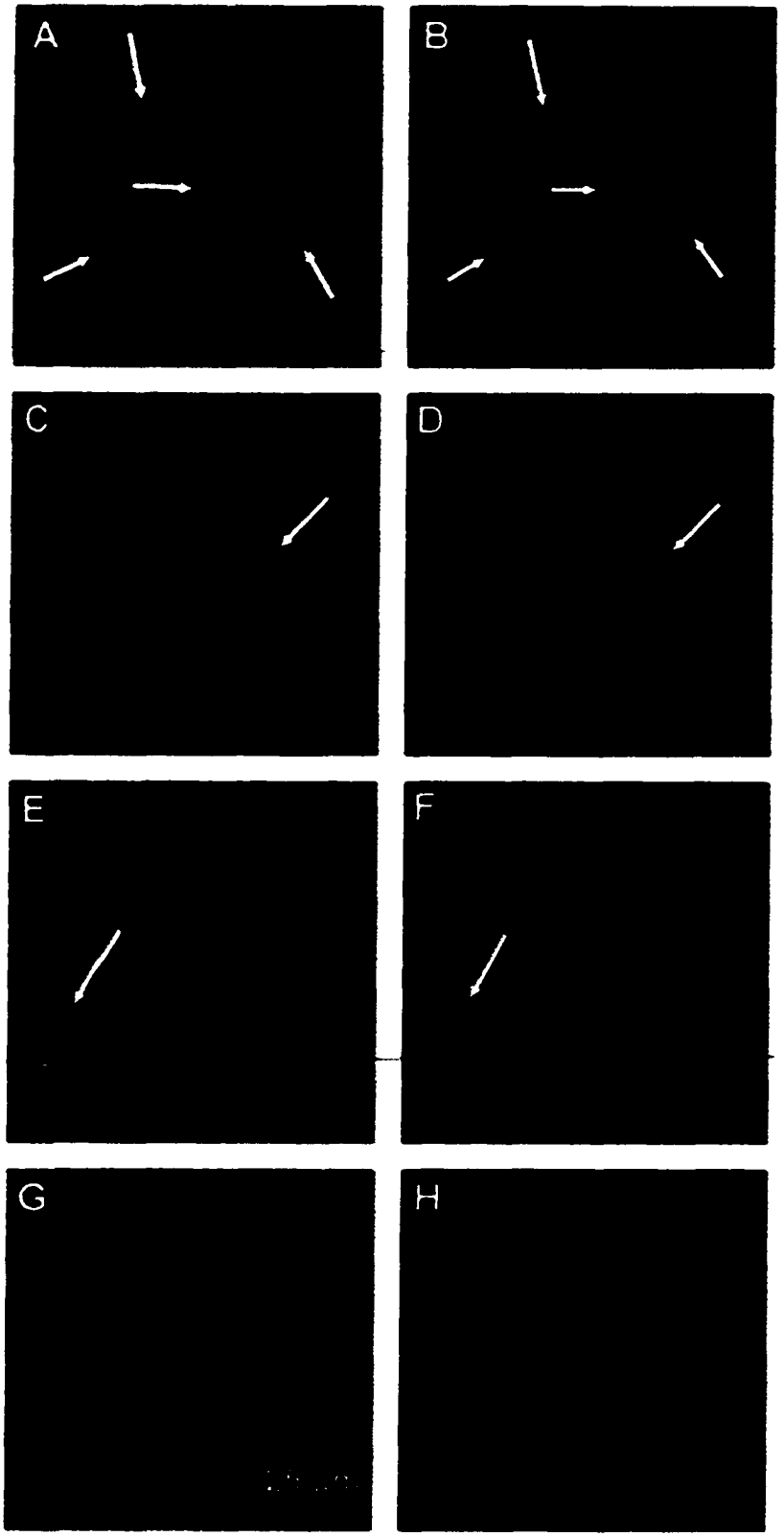

A
B
C
D
E
F
G
H

Figure 4.2: Distribution of Heat Shock Protein 90 (HSP90) following repeated dosing with VCD in rat ovary. F344 (d28) female rats were treated daily with either vehicle control (sesame oil) or VCD (80 mg/kg, i.p.) for 15 days. Rat ovaries were prepared and stained with a Hsp90 antibody. Boxes A, C, and E show the overlay of Hsp90 staining (Cy-5; RED) and nuclear DNA (YOYO-1; GREEN) while Boxes B, D, and G show the Cy-5 stain only. A and B, a control rat; C – F, VCD-treated animals. Arrows highlight primary follicles that are not expressing HSP90 from ovaries of VCD-treated rats.

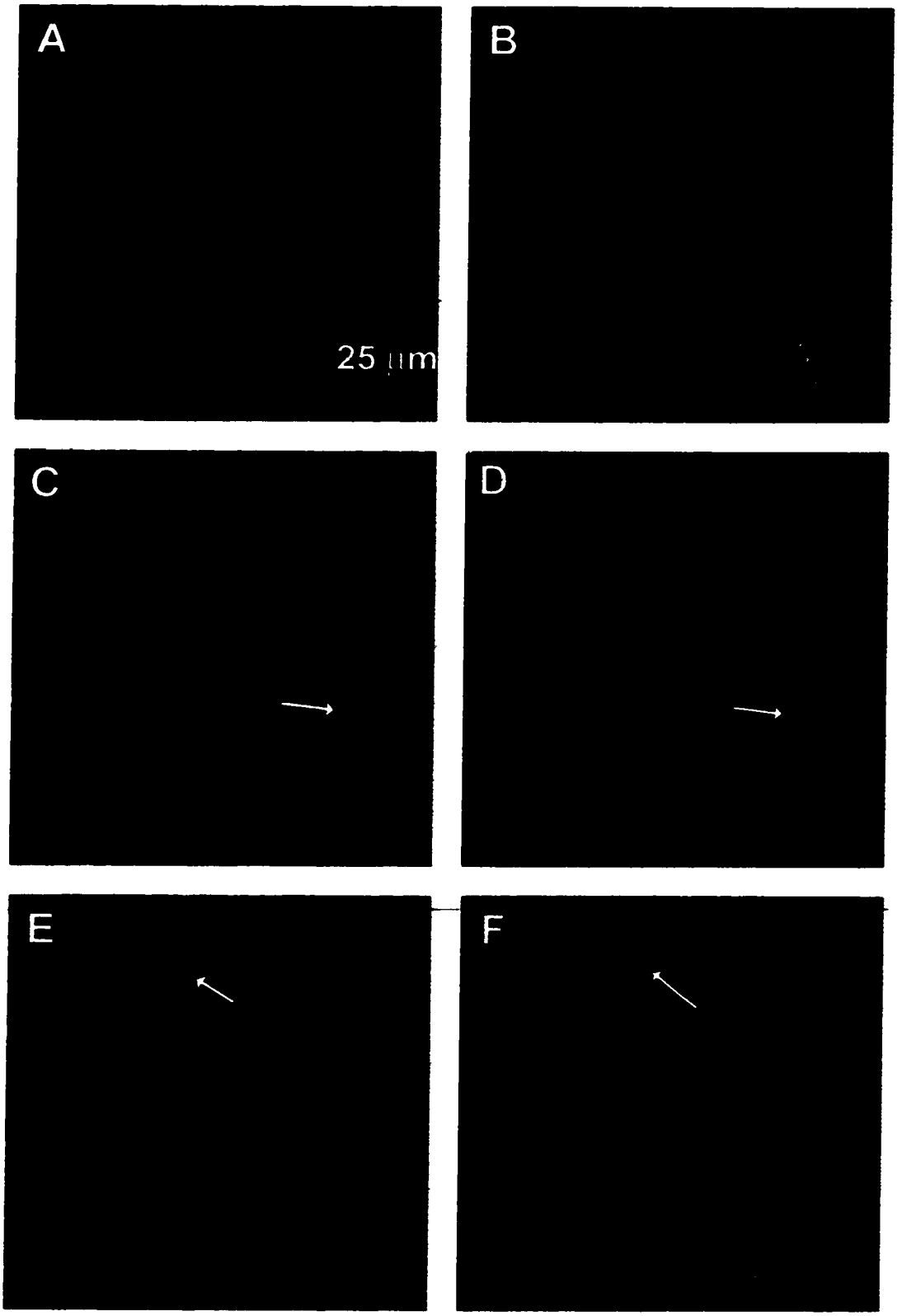

A
25 µm
B
C
D
E
F

Figure 4.3: Staining intensity of Heat Shock Protein 90 (Hsp90) in oocytes following repeated dosing with VCD in rat ovary. F344 (d28) were treated daily with either vehicle control (sesame oil) or VCD (80 mg/kg, i.p.) for 15 days. Rat ovaries were prepared, stained with a Hsp90 antibody, and analyzed by confocal microscopy. Densitometric analysis using Scion Image (NIH) was completed to calculate mean staining in the cytoplasm and nucleus of the oocyte in each follicle in arbitrary units (AU). Bars represent the mean staining for each follicle type ± SE. Means that share common superscripts are statistically the same ($p>0.05$).

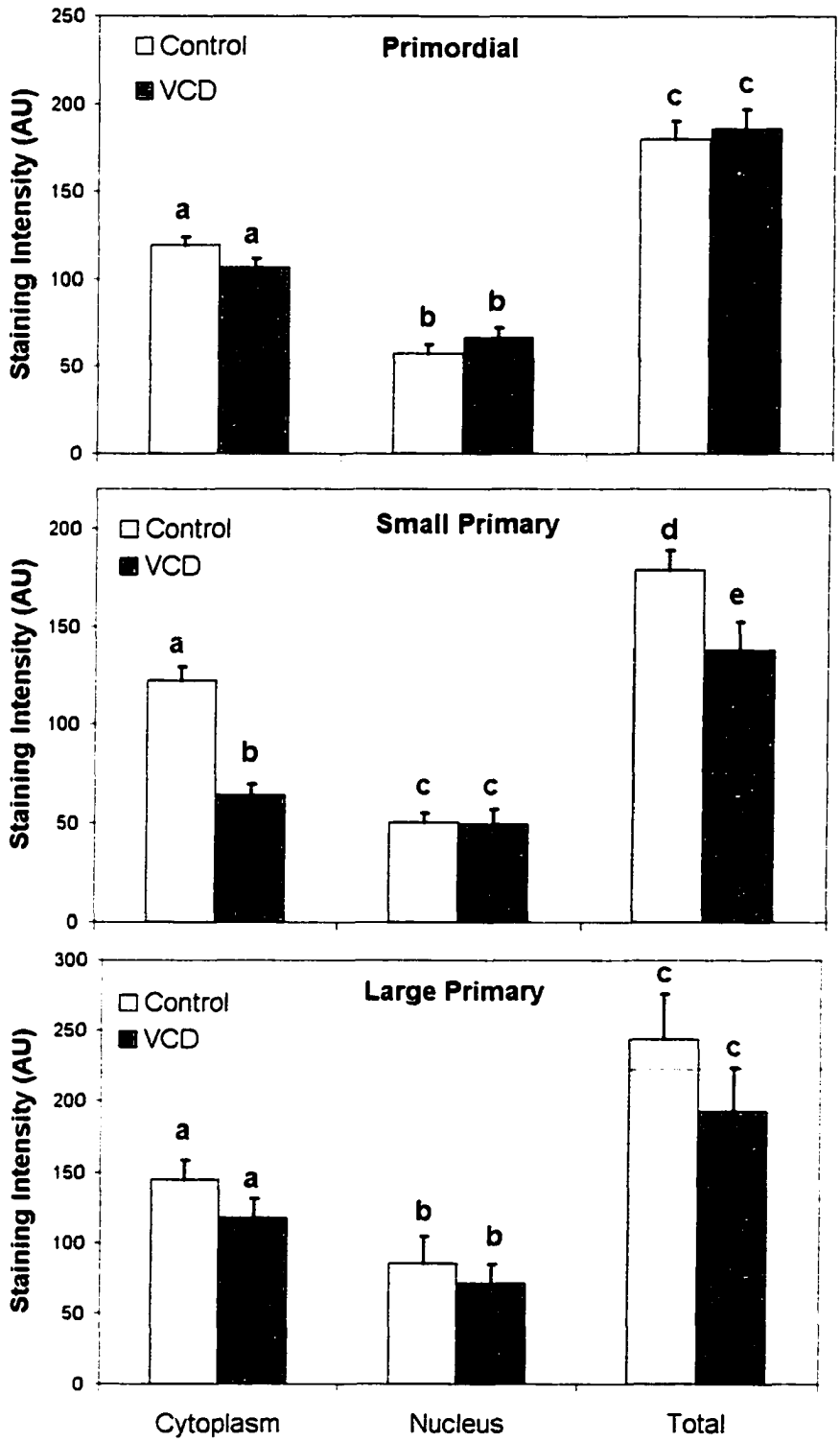
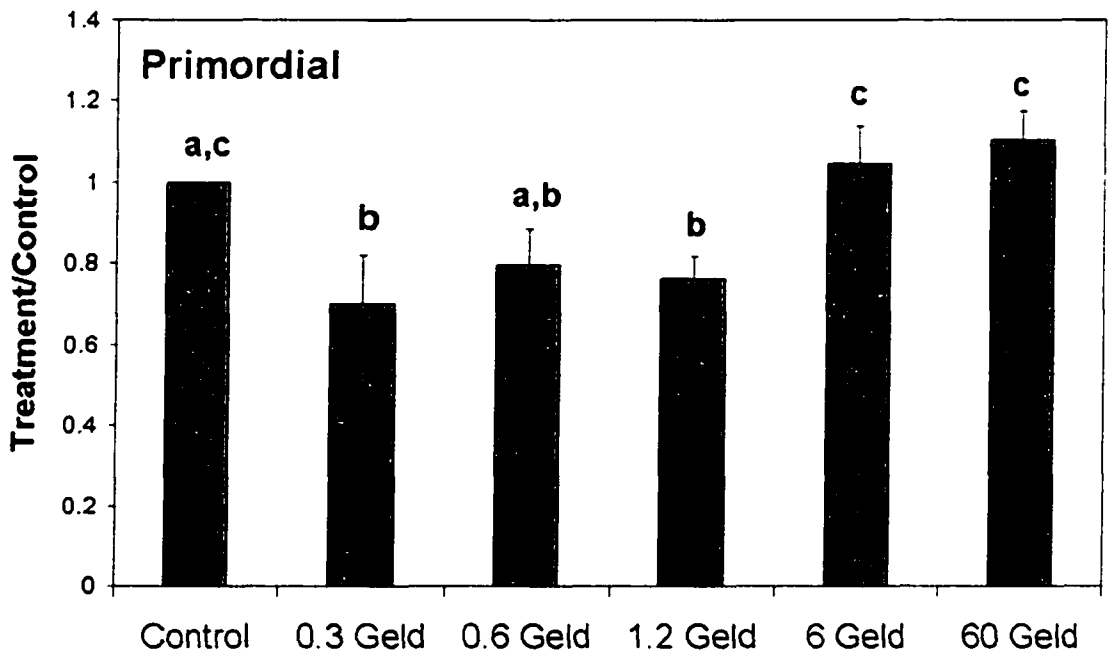
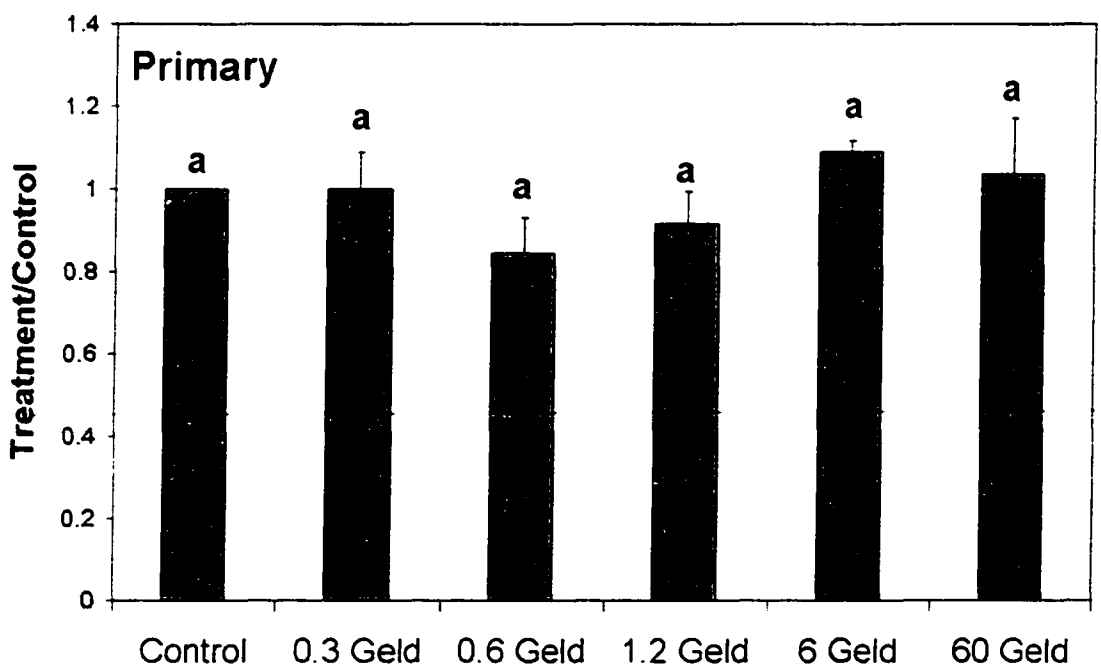

Primordial
Control
VCD
Staining Intensity (AU)
250
200
150
100
50
0
a
a
b
b
c
c
Small Primary
Control
VCD
Staining Intensity (AU)
200
150
100
50
0
a
b
c
c
d
e
Large Primary
Control
VCD
Staining Intensity (AU)
300
250
200
150
100
50
0
a
a
b
b
c
c
Cytoplasm
Nucleus
Total

Figure 4.4: Effect of geldanamycin on rat primordial and primary follicle numbers. F344 rats (d28) were dosed daily for 15 days with either vehicle control (DMSO, i.p.) or the HSP90 function inhibitor geldanamycin (Geld, 0.3-60 μg/kg, i.p.). Primordial and primary follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means that share common superscripts are statistically the same ($p>0.05$). $N \geq 4$ rats per treatment.


Primordial
Treatment/Control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a,c
b
a,b
b
c
c
Control
0.3 Geld
0.6 Geld
1.2 Geld
6 Geld
60 Geld


Primary
Treatment/Control
1.4
1.2
1
0.8
0.6
0.4
0.2
0
a
a
a
a
a
a
Control
0.3 Geld
0.6 Geld
1.2 Geld
6 Geld
60 Geld

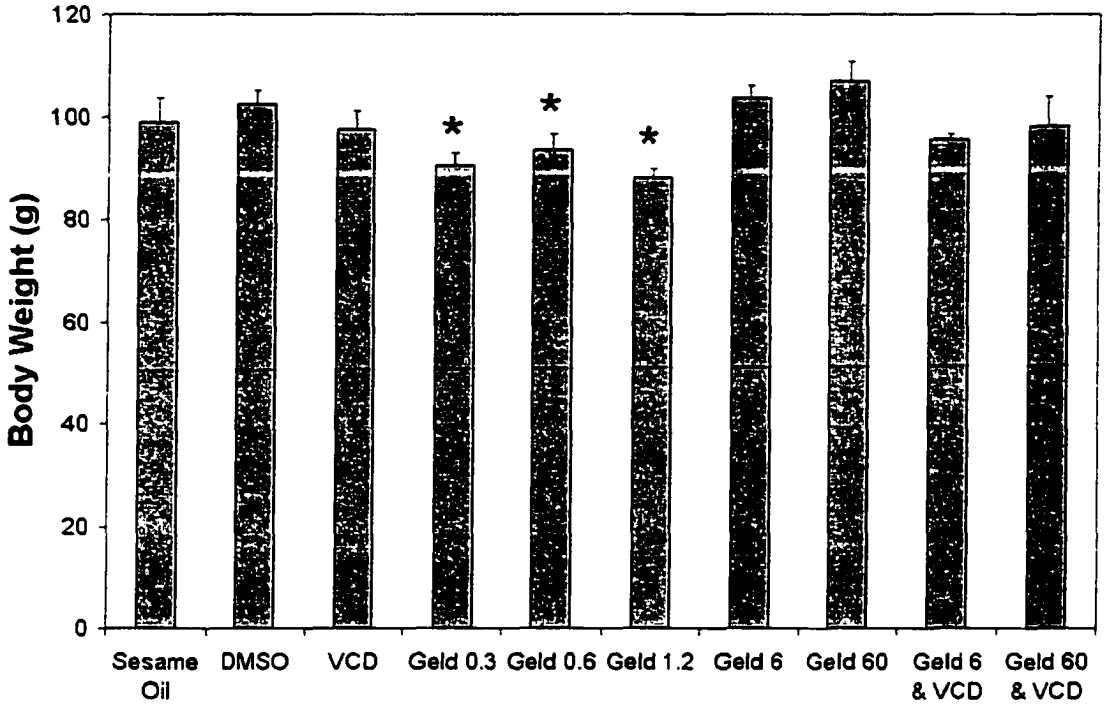
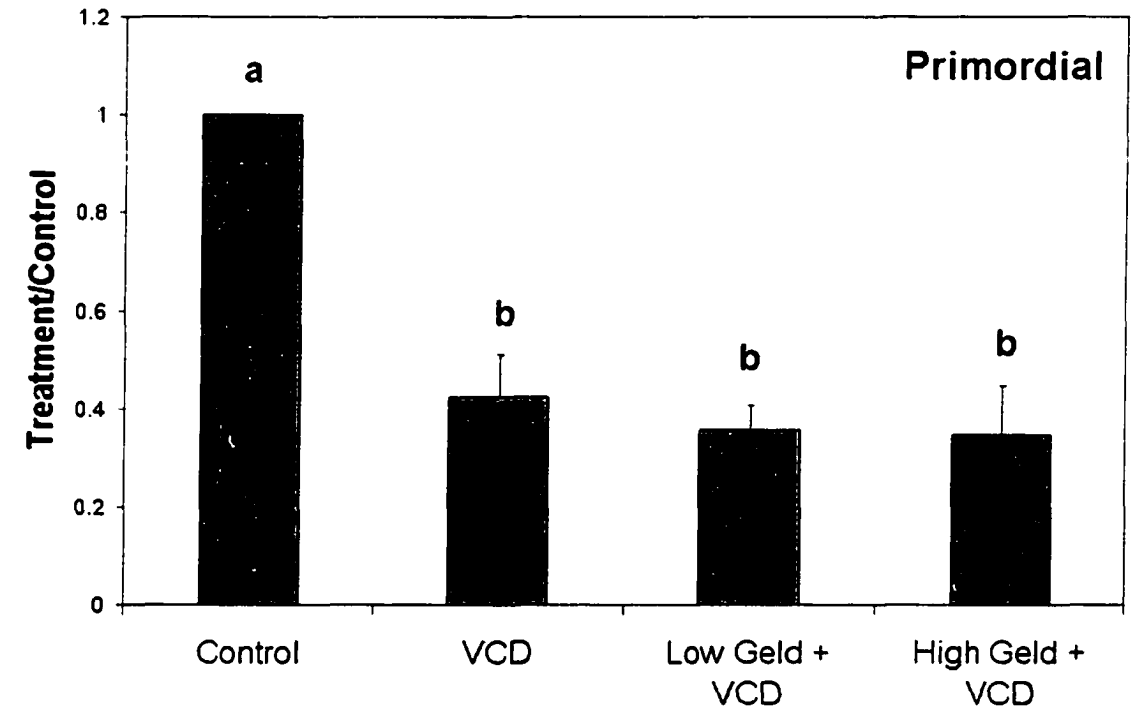
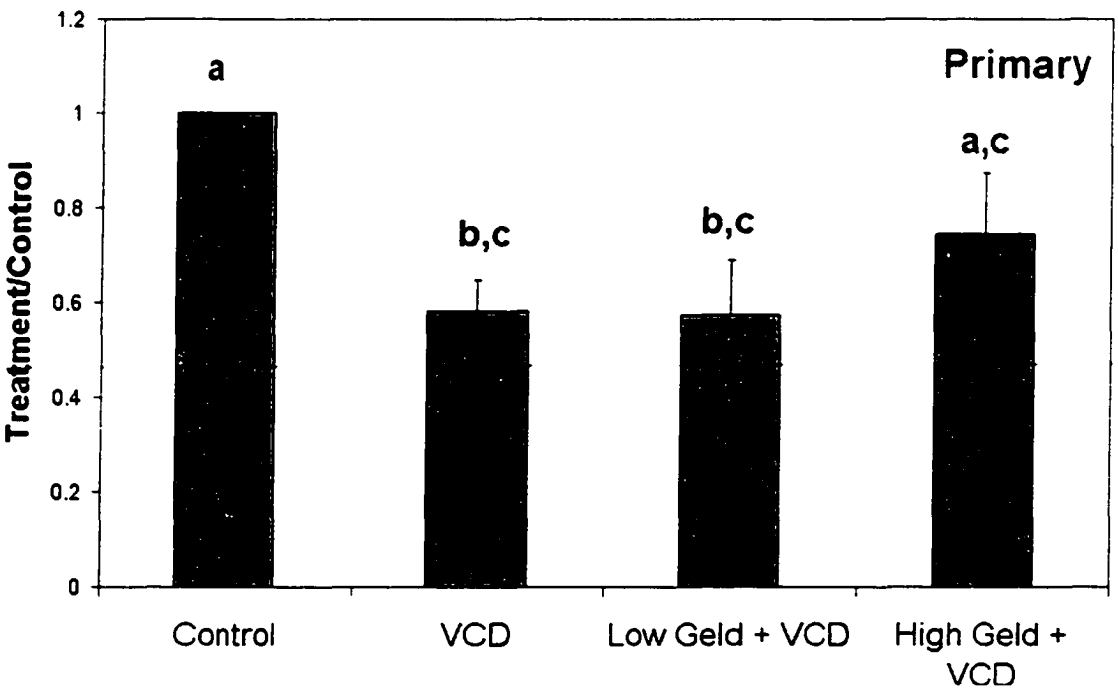


Figure 4.5: Effect of VCD and geldanamycin on body weight in F344 rats. Female rats were treated daily for 15 days with one or more of the following: sesame oil (i.p.), vehicle control (DMSO, i.p.), VCD (80 mg/kg, i.p.), or the HSP90 function inhibitor, geldanamycin (0.3-60 μg/kg, i.p.). Animals were weighed on the final day. Values are mean body weights for each treatment ± standard error; * indicates significant difference between vehicle control (DMSO) and treatment ($p<0.05$). $N \geq 4$ rats per treatment.

Figure 4.6: Effect of VCD and geldanamycin on rat primordial and primary follicle numbers. F344 rats (d28) were dosed daily for 15 days with either vehicle control (DMSO, i.p.), VCD (80 mg/kg, i.p.), or VCD and the HSP90 function inhibitor geldanamycin (Geld, 6 or 60 μg/kg, i.p.). Primordial and primary follicles were counted in every 40^{th} section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means with common superscripts are statistically similar ($p>0.05$). $N \geq 4$ rats per treatment.


Primordial
Treatment/Control
1.2
1
0.8
0.6
0.4
0.2
0
a
b
b
b
Control
VCD
Low Geld + VCD
High Geld + VCD


Primary
Treatment/Control
1.2
1
0.8
0.6
0.4
0.2
0
a
b,c
b,c
a,c
Control
VCD
Low Geld + VCD
High Geld + VCD

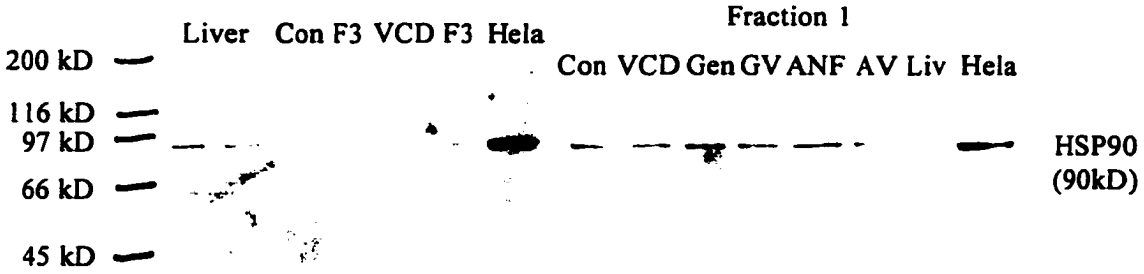


Figure 4.7: Effect of dosing with VCD on HSP90 protein expression in fraction 3 and fraction 1 follicles. F344 rats were dosed daily for 15 days with either vehicle control (sesame oil, i.p.), VCD (80 mg/kg, i.p.), genistein (Gen, 0.1 mg/kg, i.p.), genistein and VCD (GV), alpha-naphthoflavone (ANF, 80 mg/kg, i.p.), or ANF and VCD (AV). 50 μg of cellular homogenate from fraction 1 follicles, fraction 3 follicles (F3), and liver was separated by SDS-PAGE and transferred to nitrocellulose. HSP90 protein was detected with a HSP90 antibody and a HRP-conjugated secondary antibody. Heat-shocked Hela cells were run as a positive control in each experiment. Western blotting showed a single band at the predicted size of 90 kD for all tissues.

Table 4.1: Localization of ERα, ERβ, AhR, and HSP90 in small pre-antral rat follicles.

	Primordial				Small Primary				Large Primary			
	Oocyte		Granulosa		Oocyte		Granulosa		Oocyte		Granulosa	
	Cyt	Nuc	Cyt	Nuc	Cyt	Nuc	Cyt	Nuc	Cyt	Nuc	Cyt	Nuc
ERα	-	+	-	-	-	+	-	-	-	+	-	-
ERβ	+	+	+	+	+	+	+	+	+	+	+	+
AhR	-	+	-	+	-	+	-	+	-	+	-	+
HSP90	+	+	-	-	+	+	-	-	+	+	-	-

Cyt = cytoplasm

Nuc = nucleus

+ = positive staining for protein

- = negative staining for protein

DISCUSSION

HSP90 was expressed specifically in oocytes in all sizes of follicles. Little staining was seen in other ovarian compartments. This suggests a specialized role of HSP90 in the oocyte, however it is unclear what this role may be. In bovine dominant follicles, HSP90 has been found to act as an aromatase activity inhibitor (Driancourt *et al.*, 1999). Primordial and primary follicles do not produce the enzyme aromatase or the steroid estradiol (via aromatase) in this early stage of development, therefore, it seems unlikely that HSP90 is expressed in these small follicles as an aromatase inhibitor (Channing *et al.*, 1980; Inkster and Brodie, 1991). The study by Driancourt *et al.* (1999) also found a decrease in HSP90 protein in atretic follicles as compared to healthy follicles. Therefore, a loss of HSP90 could be associated with apoptosis in follicles. VCD accelerates the natural rate of atresia in primordial and primary follicles (Borman *et al.*, 1999). VCD also decreased expression of HSP90 in small primary follicles. This suggests that VCD modulation of HSP90 expression may increase follicular loss.

HSP90 is considered to be a cytoplasmic protein, however in this study, HSP90 was seen in the nucleus of some oocytes (Buchner, 1999). This finding is in agreement with previous research which found a translocation of HSP90 to the nucleus (Berbers *et al.*, 1988). Additionally, in adult rats, HSP90 has been localized to the oocyte nuclei in pre-ovulatory follicles (Ohsako *et al.*, 1995). HSP90 is a steroid hormone receptor chaperone protein (Buchner, 1999). Since most steroid hormone receptors are located in the nucleus, HSP90 may be localized within the nucleus to serve a physiological

function. However, in many other cell types which contain steroid hormone receptors located in the nucleus, HSP90 is not seen in the nucleus (Tuohimaa *et al.*, 1993). Therefore, expression of HSP90 in the oocyte nucleus could indicate a novel, unique role of this protein in the follicle.

Geldanamycin is an anti-tumor, anti-inflammatory antibiotic (Schneider *et al.*, 1996). Geldanamycin is also known to be a protein kinase inhibitor and inhibit HSP90 function (Dunlap *et al.*, 1999; Roe *et al.*, 1999). Geldanamycin binds HSP90 and blocks HSP90 ATPase activity, which is required for its *in vivo* function (Roe *et al.*, 1999). Subsequently, HSP90 is unable to bind steroid hormone receptors and serve its endogenous chaperone function (Segnitz and Gehring, 1997). If HSP90 is serving an anti-atretic role in the ovary, then inhibition of HSP90 activity would enhance follicular loss. Treatment of rats with low doses of geldanamycin (0.3 and 1.2 μg/kg) showed a loss of primordial follicles. However, this effect was not seen at higher doses of geldanamycin (6 or 60 μg/kg). Along with this follicular effect, treatment with low, but not high, doses of geldanamycin also decreased body weight of rats. Therefore, it is unclear if the follicular loss is due to generalized toxicity that is affecting the entire animal or if there is a specialized dose-response curve. This study should be repeated to see if low doses of geldanamycin are able to induce follicular loss. Other tissue weights (i.e. kidney, liver, adrenal glands, spleen, etc.) should be examined to see if geldanamycin is inducing a systematic effect on rats. Additionally, it would be useful to determine if geldanamycin is able to exert its inhibitory effects on HSP90 at the level of the follicle.

If VCD is reducing HSP90 protein expression and geldanamycin is reducing HSP90 function, then cotreatment of rats with these chemicals should potentiate follicle depletion. Geldanamycin dosing at 6 or 60 μg/kg did not alter VCD-induced follicle loss. However, since geldanamycin did not induce follicular loss at these doses when given alone, it seems unlikely that it would alter VCD-induced toxicity. Future studies using the lower doses of geldanamycin (0.3-1.2 μg/kg) along with VCD are warranted.

Western analysis of HSP90 protein showed that treatment with VCD did not alter HSP90 protein levels in VCD-targeted fraction 1 follicles or non-VCD-targeted fraction 3 follicles. However, confocal microscopy and staining analysis showed that VCD dosing decreased HSP90 protein specifically in the oocyte in small primary follicles. Fraction 1 follicles contain a heterogeneous mix of granulosa cells and oocytes in primordial, small and large primary, and small secondary follicles. Therefore, the specific loss of HSP90 protein in the oocyte of small primary follicles as measured by immunohistochemistry was likely overwhelmed by the other non-responding cells when analyzed by western blotting.

Dosing with ER agonists and AhR antagonists protect primary follicles from VCD-induced loss (Chapters 2 and 3). In this study, dosing with analogs of the ER and AhR did not alter HSP90 protein expression in VCD-targeted fraction one follicles. Therefore, it seems unlikely that modulation of HSP90 expression is part of the protective effects seen with either of these receptors. Additionally, based on the protein localization of the ER, AhR, and HSP90, it is unclear how these proteins may be interacting (Table 4.1). The oocyte nucleus appears to be the only site in the follicle in which all three of

the proteins are expressed. It seems unusual that HSP90 is not seen in granulosa cells expressing the ER and AhR since HSP90 has a defined role in nuclear receptor signaling. Therefore, the ER and AhR are either able to bind ligands and mediate transcription in the absence of HSP90 or they are using alternative pathways to mediate their physiologic effects. Subsequently, these receptors may be working via non-genomic or undiscovered pathways.

Taken together, VCD selectively depletes HSP90 in the oocyte cytoplasm of small primary follicles. If geldanamycin is exerting its anti-HSP90 function, than the loss of function of HSP90 is not enough to induce follicle loss. Therefore, HSP90 may play a peripheral role in toxicant-induced ovotoxicity or reflect a generalized response to cell stress. Additionally, HSP90 is co-localized with ERβ and AhR only in the oocyte nucleus and HSP90 is not regulated by analogs of these receptors, suggesting that HSP90 is not serving as a chaperone protein in ovarian follicles. Therefore, the specific role of HSP90 in primordial and primary follicles is yet to be determined.

CHAPTER 5

CONCLUSION

The overall purpose of these studies was to investigate potential roles of several nuclear receptors in VCD-induced follicle loss. This research focused on the estrogen receptor and aryl hydrocarbon receptor. Activation of ER and inhibition of the AhR protected small pre-antral follicles from VCD-induced ovotoxicity, however no evidence was seen that these receptors are interacting with each other. Additionally, these studies examined a possible role of a chaperone protein associated with both of these nuclear receptors, HSP90. HSP90 protein expression was affected during VCD-induced ovotoxicity, however, whether this relates to its role as a receptor chaperone was not demonstrated.

Treatment with high doses of estradiol selectively protects primary follicles from VCD-induced follicle loss. This protective effect was modulated by the estrogen receptor via a reduction in caspase-3-induced apoptosis. Additionally, there was a unique expression of ERβ in primordial and primary follicles. ERβ protein was seen in the cytoplasm of the oocyte and granulosa cells in these smallest follicles (primordial and primary), while it was compartmentalized to the nucleus in larger follicles (large pre-antral and antral). Another form of ERβ (ERβ2) has been found in rats (Petersen *et al.*, 1998). It is expressed at equal levels to ERβ1 in the ovary and recognizes the same consensus estrogen response element. However, ERβ2 binds estradiol with a lower affinity than ERβ1 and requires 1000-fold higher doses of estradiol to activate

transcription (Petersen *et al.*, 1998). Additionally, ERβ2 has been shown to bind both genistein and 4-hydroxytamoxifen (Petersen *et al.*, 1998). Therefore, perhaps the cytosolic ERβ protein detected in primordial and primary follicles is ERβ2. Since ERβ2 requires higher doses of E_2 to regulate transcription, this could explain why pharmacological doses of E_2 were required to protect primary follicles from VCD-induced loss if this effect is mediated via ERβ2.

Treatment with estradiol decreased caspase-3 activity, likely via a receptor mediated-mechanism. The estrogen receptor exerts its physiologic function by regulating transcription of specific genes. Estradiol has been found to alter the expression of many of the *bcl-2*-related genes. Expression of mRNA encoding the cell survival genes *bcl-2* and *bcl-X_L* is upregulated by 17β-estradiol in MCF-7 cells, neural cells, and the corpus luteum (Goodman *et al.*, 1998; Wang and Phang, 1995; Singer *et al.*, 1998; Garcia-Segura *et al.*, 1998; Gollapudi and Oblinger, 1999a,b; Pike, 1999). Several putative estrogen-responsive sites are present in the *bcl-2* promoter (Teixeira *et al.*, 1995). Additionally, in neuroblastoma and monoblastoid cells, estradiol has been found to decrease expression of Nip2, a negative regulator of Bcl-2 (Garnier *et al.*, 1997; Vegeto *et al.*, 1999). Treatment with estradiol has also been shown to decrease expression of the cell death enhancer Bad in neuronal cells (Gollapudi and Oblinger, 1999b). Estradiol has not been shown to alter expression of another cell death enhancer, Bax, in sensory neurons (Patrone *et al.*, 1999). Therefore, by altering expression of the members of the *bcl-2* family of proto-oncogenes, estradiol can reduce apoptosis. Research by Hu *et al.* (2001a) has demonstrated that repeated treatment with VCD increases Bad protein

expression and implicates the *bcl-2* family of genes as important mediators in VCD-induced apoptosis, eventually leading to caspase-3 activity induction (Figure 5.1; Hu *et al.*, 2001b). Taken together, estradiol may be protecting primary follicles from VCD-induced ovotoxicity by altering expression of *bcl-2* family genes, thereby reducing apoptosis (Figure 5.2).

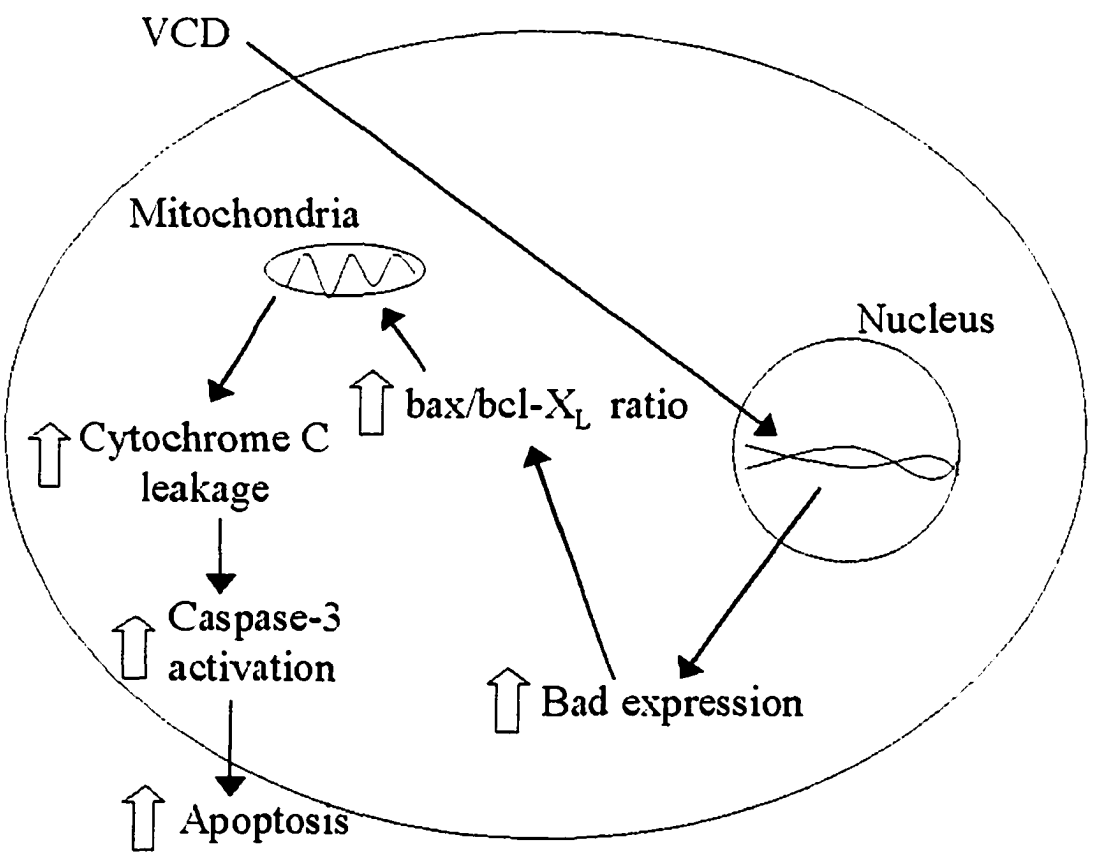


Figure 5.1: VCD-mediated initiation of apoptosis via modulation of the *bcl-2* gene family in rat small pre-antral follicles. Following repeated dosing with VCD, expression of the cell death enhancer protein, Bad, is increased. Bad then sequesters bcl-X_L, and increases the Bax/Bcl-X_L ratio at the mitochondria. This increases the formation of Bax/Bax dimers and subsequently, the Bax homodimer is able to initiate its detrimental effects to the mitochondrial membrane, leading to cytochrome *c* leakage. This pathway is a combination of data from Hu *et al.* (2001a) and Hu *et al.* (2001b).

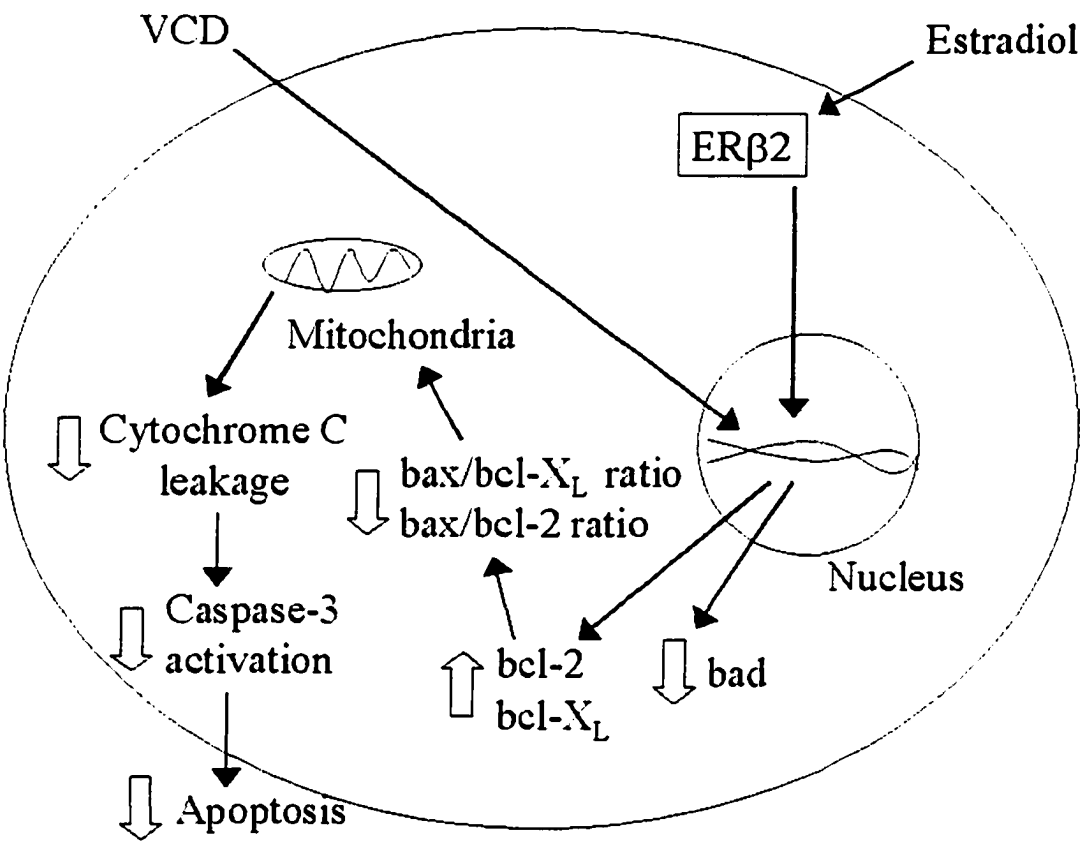


Figure 5.2: Proposed mechanism of protection by estradiol against VCD-induced follicle loss in primary follicles. Estradiol binds to a cytoplasmic form of ERβ, ERβ2, in primary follicles and alters expression of *bcl-2* family members to favor cell survival. This negates VCD-induced alterations in these genes, thereby reducing VCD-induced caspase-3 activity and protecting follicles from depletion.

The AhR may also be involved in VCD-induced acceleration of follicle loss. Treatment of rats with an AhR antagonist slowed down the normal rate of atresia as compared to control and additionally, prevented VCD-induced follicle loss, as evidenced by follicle counts and caspase-3 activity. VCD was also shown to upregulate expression of mRNA encoding the *AhR*. Althought there is no known endogenous ligand for the AhR to date, the AhR has been shown to modulate cellular functions without xenobiotic stimulation (Ma and Whitlock, 1996; Reiners and Clift, 1999). For example, the AhR has been reported to increase apoptosis in the absence of known exogenous AhR ligands. Therefore, the AhR is involved in the control of physiological cell death either in the presence of an endogenous ligand or without ligand activation (Reiners and Clift, 1999). The cell death enhancer gene, *bax*, has two AhR response element consensus sequences (XREs) in its promoter region (Matikainen *et al.*, 2001). Subsequently, the AhR, in part, could increase apoptosis due to increased expression of *bax*.

The regulation of atresia, which occurs via apoptosis, is not completely understood. Since AhR-deficient mice have altered atresia, the AhR and its role in apoptosis in the absence of an exogenous ligand could potentially be involved in the control of atresia. As AhR-deficient mice still undergo atresia, the AhR is only one part of the large and complex process of follicle regulation. VCD also modulates the rate of atresia. Thus, the AhR and VCD affect the same endpoint. It is unclear whether the AhR and VCD pathways that lead to atresia are directly interacting. VCD dosing induced follicle loss in mice lacking the AhR gene, even though VCD upregulates expression of *AhR* mRNA in rats. Dosing with VCD also increases expression of mRNA encoding *bax*

in rats (Springer *et al.*, 1996b). By increasing *AhR* expression, VCD could cause AhR-driven *bax* expression. This could lead to apoptosis in the absence of an exogenous AhR ligand (Figure 5.3). By this reasoning, activation of classical *AhR* genes would not necessarily be regulated by VCD treatment, since VCD would not be acting as an exogenous ligand.

Finally, the nuclear receptor chaperone protein HSP90 may play a potential role in VCD-induced follicle loss. HSP90 is only co-localized with expression of ER (α or β) or AhR in the oocyte nucleus. Thus, potential interactions would likely occur at that site. VCD reduced HSP90 protein in the cytoplasm of oocytes in small primary follicles. Whether this reduction relates to an interaction between VCD, ER, and AhR was not demonstrated. Analogs of these receptors did not alter expression of HSP90. Taken together, these observations suggest that HSP90 is not involved with VCD toxicity in its role as a nuclear receptor chaperone.

Since VCD dosing depletes expression of HSP90 in small primary follicles and since depletion of HSP90 is associated with an increase in apoptosis, HSP90 may play a role in VCD-induced apoptosis (Lee *et al.*, 2001; Hostein *et al.*, 2001; Pandey *et al.*, 2000). In the induction of apoptosis, cytochrome *c* release from the mitochondria causes the formation of an Apaf-1-caspase-9 apoptosome (Li *et al.*, 1997). This subsequently activates procaspase-3 and protein degration (Li *et al.*, 1997). HSP90 forms a cytosolic complex with Apaf-1 and thereby inhibits formation of the Apaf-1-caspase-9 apoptosome and prevents procaspase-3 activation (Pandey *et al.*, 2000). Therefore, HSP90 plays a direct role in cytochrome *c*-mediated induction of apoptosis. Treatment with VCD is

known to cause cytochrome *c* release into the cytosol in small primary follicles and as well, activation of pro-caspase-3 in small pre-antral follicles (Hu *et al.*, 2001a,b). Therefore, one pathway potentially employed by VCD to target small primary follicles is a depletion of HSP90 (Figure 5.4).

In summary, the ER plays a protective role in VCD-induced follicle loss, while HSP90 and AhR appear to be regulated by VCD, but may not be involved in the direct pathway by which VCD induces apoptosis. Future studies should help elucidate more specifically the specific mechanism(s) these proteins play in the modulation of apoptosis and atresia in small pre-antral follicles.

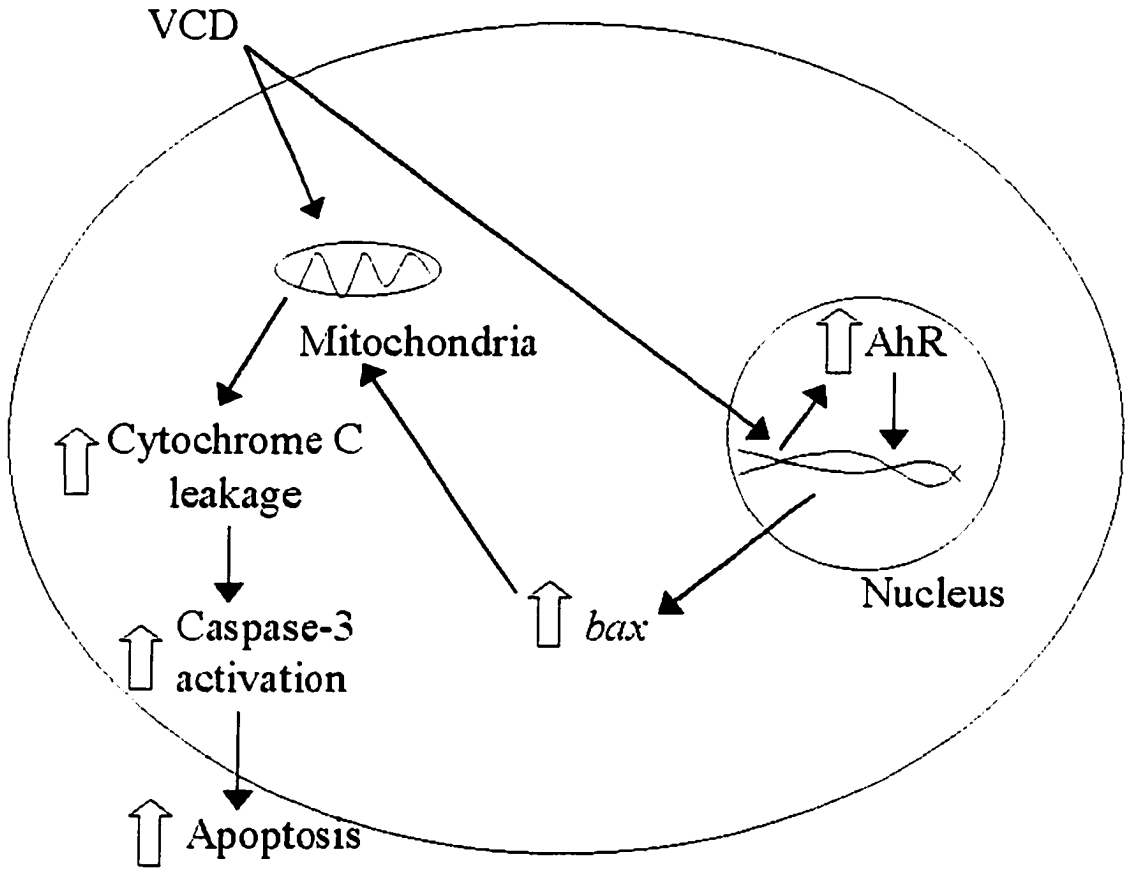


Figure 5.3: Proposed mechanism of AhR-driven apoptosis in small pre-antral follicles. The AhR is able to mediate apoptosis in the absence of an exogenous ligand. Dosing with VCD increases expression of *AhR* and *bax* mRNA and induces Caspase-3 activity. Increased expression of the *AhR* could be the mechanism of increased *bax* expression since the *bax* promoter contains two XREs. In the hypothesis of Bax-mediated Cytochrome *C* release, increased *bax* expression will eventually lead to activation of the caspases and apoptosis.

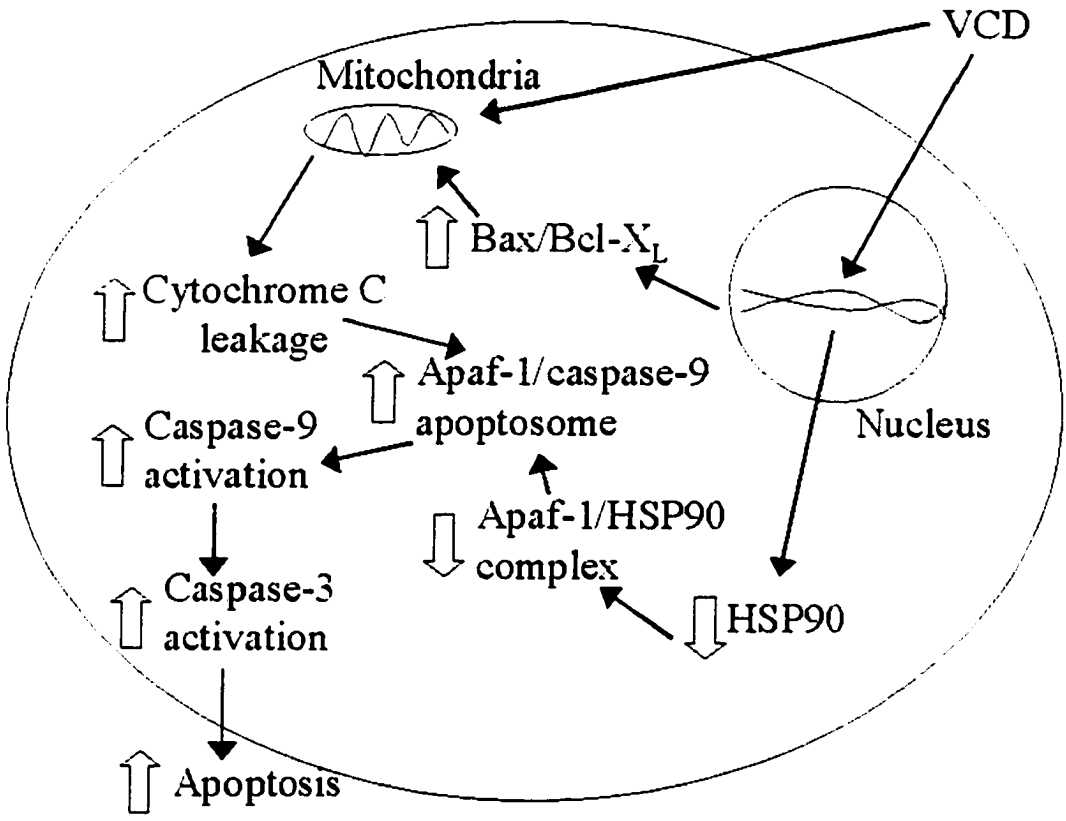


Figure 5.4: Proposed mechanism of VCD-induced follicle loss involving depletion of HSP90. Repeated treatment with VCD decreases HSP90 protein in small primary follicle oocytes. A depletion of HSP90 prevents it from chaperoning Apaf-1 and allows Apaf-1 to form a complex with caspase-9 in the presence of cytochrome *c*. Dosing with VCD regulates members of the *bcl-2* family in order to favor increased cell death (increased Bax/Bcl-X_L ratio). Bax is then able to cause cytochrome *c* leakage from the mitochondria and activation of caspase-9/Apaf-1 complex. Active caspase-9 subsequently activates procaspase-3, a point of no return in the apoptosis cascade.

APPENDIX A

SUPPLEMENTAL RESULTS

A.1: Data for Chapter Two

A.2: Data for Chapter Three

A.3: Data for Chapter Four

A.4: Effect of VCD on Sprague-Dawley Rats

APPENDIX A.1

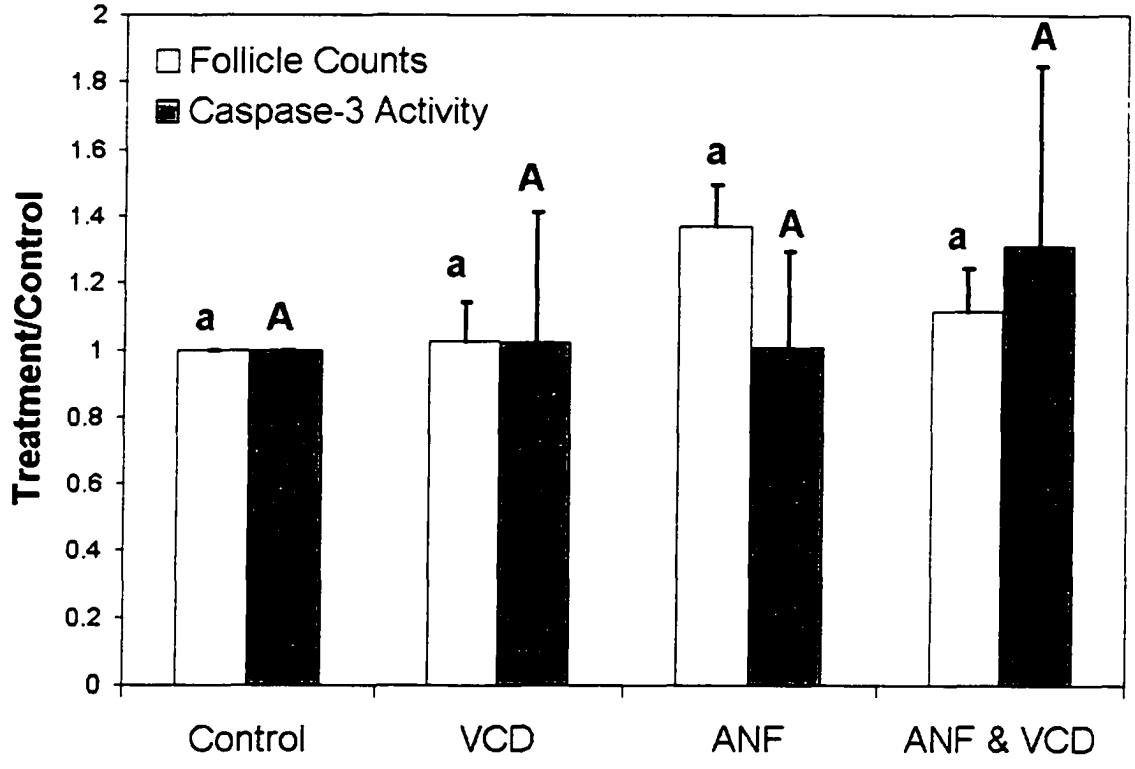


Figure A.1: Effect of dosing with VCD and an AhR antagonist, alpha-naphthoflavone (ANF), on rat large pre-antral ovarian follicle numbers and caspase-3-like activity. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), ANF (80 mg/kg, i.p.) or VCD and ANF. Secondary follicles were counted in every 40th section of ovaries as described in materials and methods. Caspase-3-like activity was measured in isolated fraction 2 follicles (100-250 μm) as described in the methods. Open bars; mean follicles counted in each ovary ± standard error (N=7-9 rats per treatment). Shaded bars; mean fluorescence as a measure of caspase-3-like activity ± standard error (N=3); means with common superscripts are statistically the same ($p>0.05$).

APPENDIX A.1

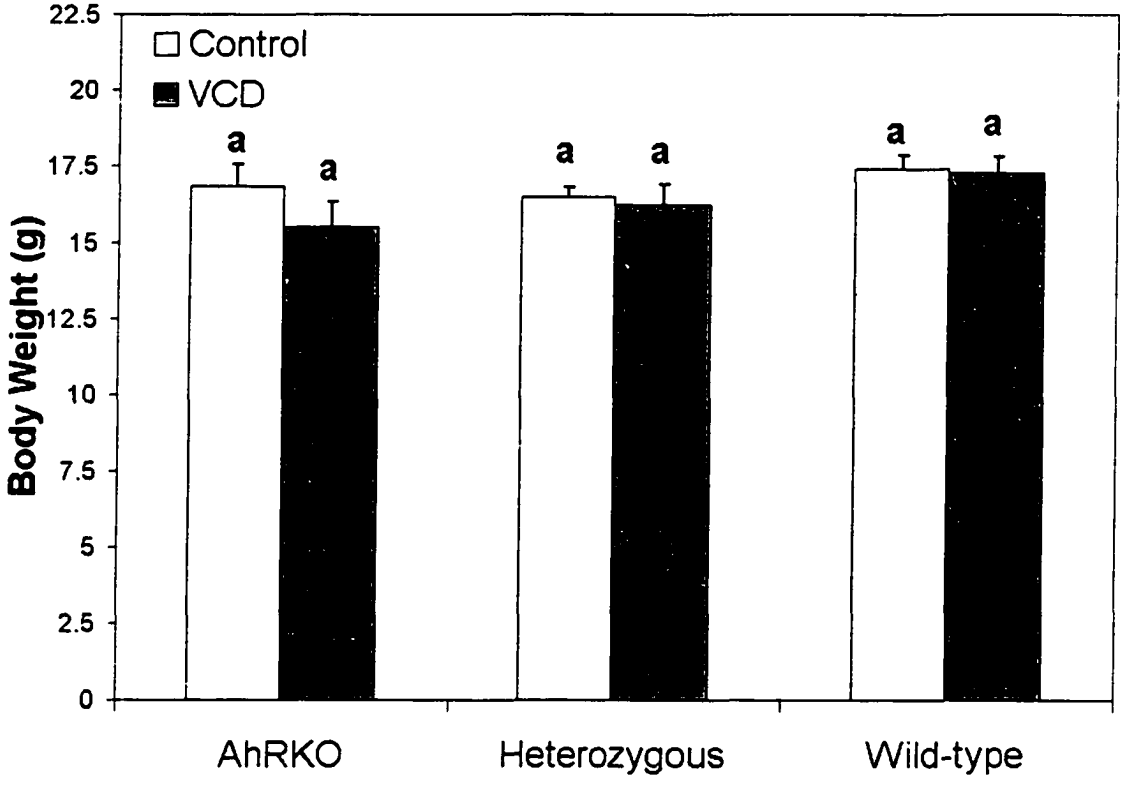


Figure A.2: Effect of VCD on body weights of AhRKO, heterozygous, and wild-type mice. Female mice were treated daily for 15 days with either vehicle control (sesame oil, i.p.) or VCD (80 mg/kg, i.p.). Animals were weighed on the final day. Values are mean body weights for each treatment ± standard error; means with common superscripts are statistically similar ($p>0.05$). N = 3-5 mice per treatment.

APPENDIX A.1

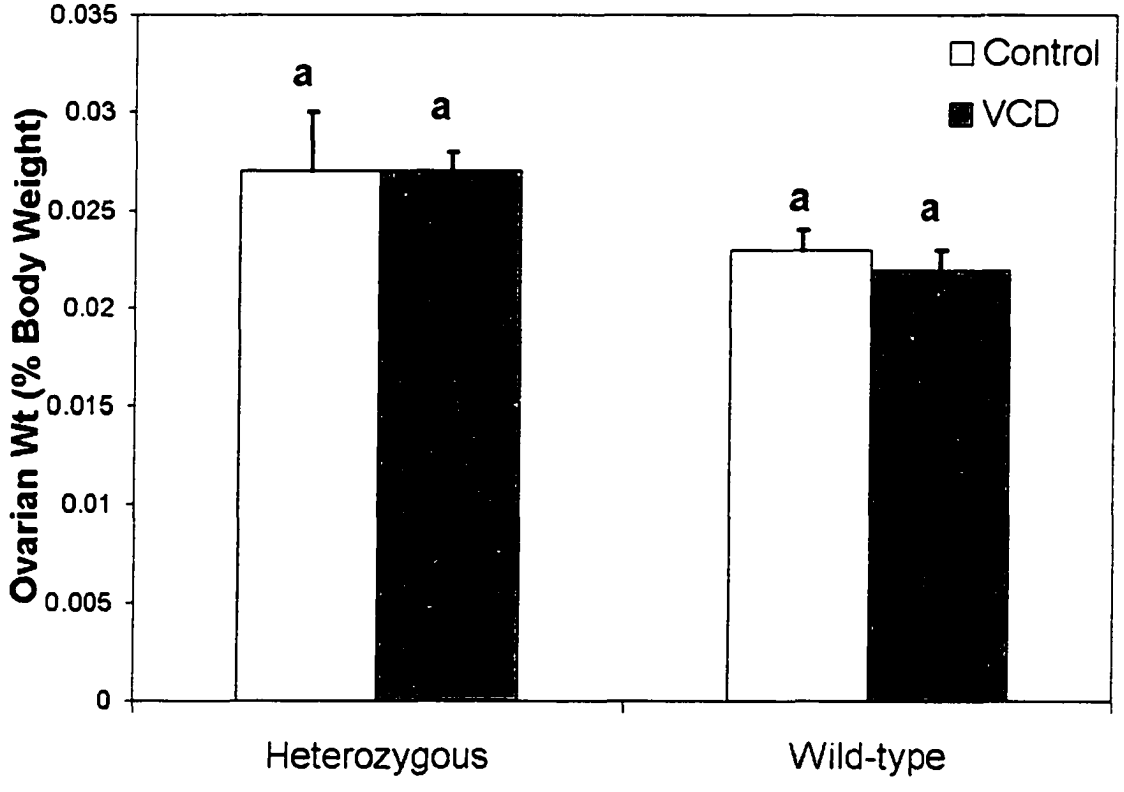


Figure A.3: Effect of VCD on ovarian weight in AhR heterozygous and wild-type mice. Female mice were treated daily for 15 days with either vehicle control (sesame oil, i.p.) or VCD (80 mg/kg, i.p.). Animals were weighed on the final day. Ovaries were collected and weighed. Values are mean ovarian weight as a percent of body weight for each treatment ± standard error. Means with similar superscripts are statistically the same ($p>0.05$). N = 4-10 mice per treatment.

APPENDIX A.2

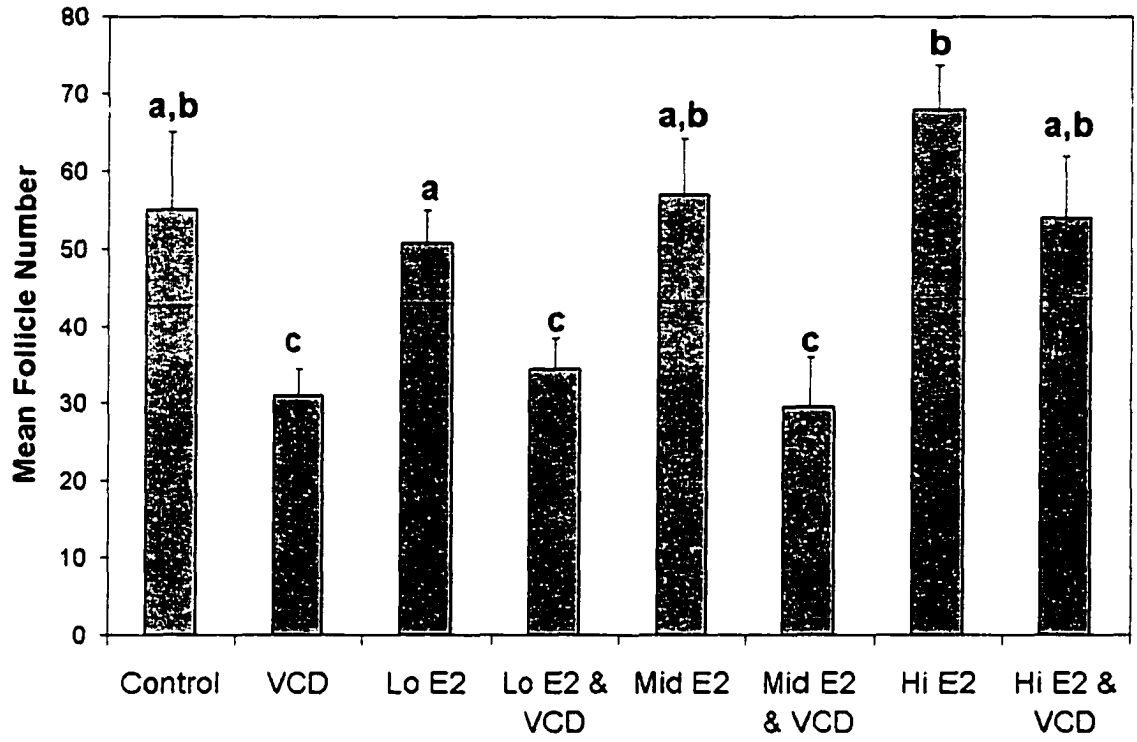


Figure A.4: Primary follicle number dose response for estradiol. F344 rats (d28) were dosed daily for 15 days with one or more of the following treatments: vehicle control (sesame oil), VCD (80 mg/kg, i.p.), Hi E_2 (0.1 mg/kg, s.c.), Mid E_2 (0.01 mg/kg, s.c.), or Lo E_2 (0.001 mg/kg, s.c.). Primary follicles were counted in every 40^{th} section as described in Methods. Values are mean total follicles counted in each ovary ± standard error; means with similar superscripts are statistically the same ($p>0.05$). $N \geq 4$ rats per treatment.

APPENDIX A.2

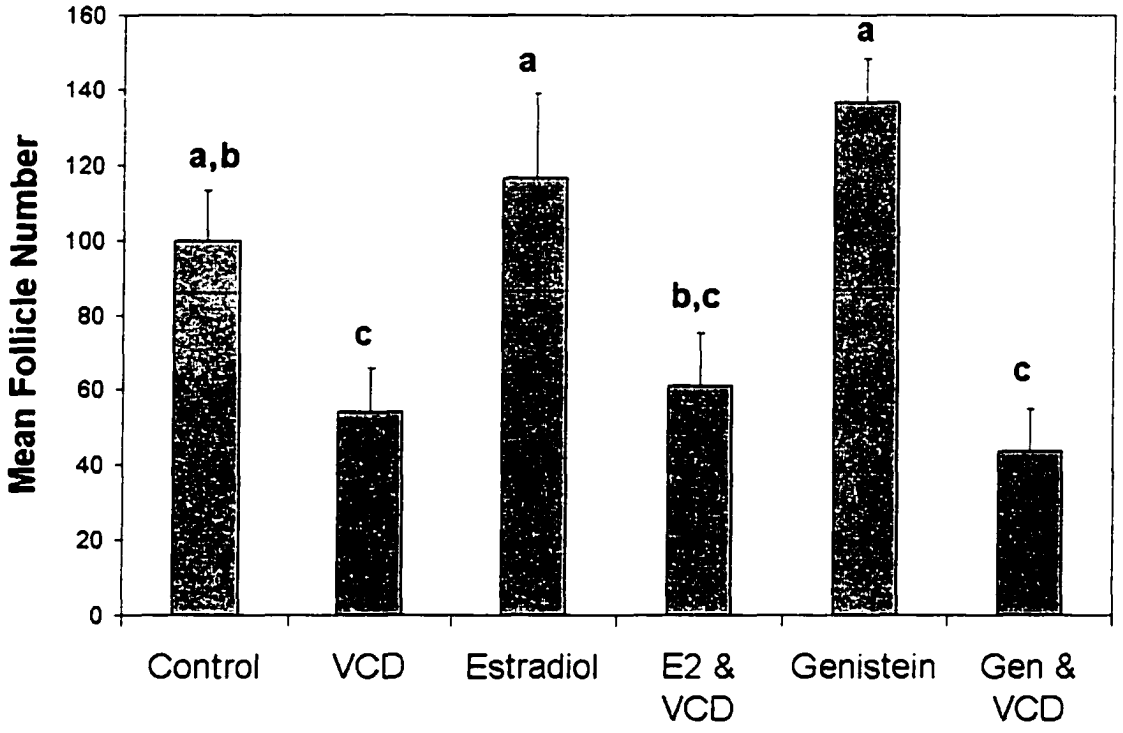


Figure A.5: Effect of dosing with VCD, 17β-estradiol (E_2), and an ER agonist, genistein (Gen), on rat primordial follicles. F344 rats (d28) were dosed daily for 15 days with one or more of the following treatments: vehicle control (sesame oil), VCD (80 mg/kg, i.p.), E_2 (0.1 mg/kg, s.c.), or genistein (0.1 mg/kg, i.p.). Primordial follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary ± standard error; means with the same superscript are statistically similar ($p>0.05$). $N \geq 4$ rats per treatment.

APPENDIX A.2

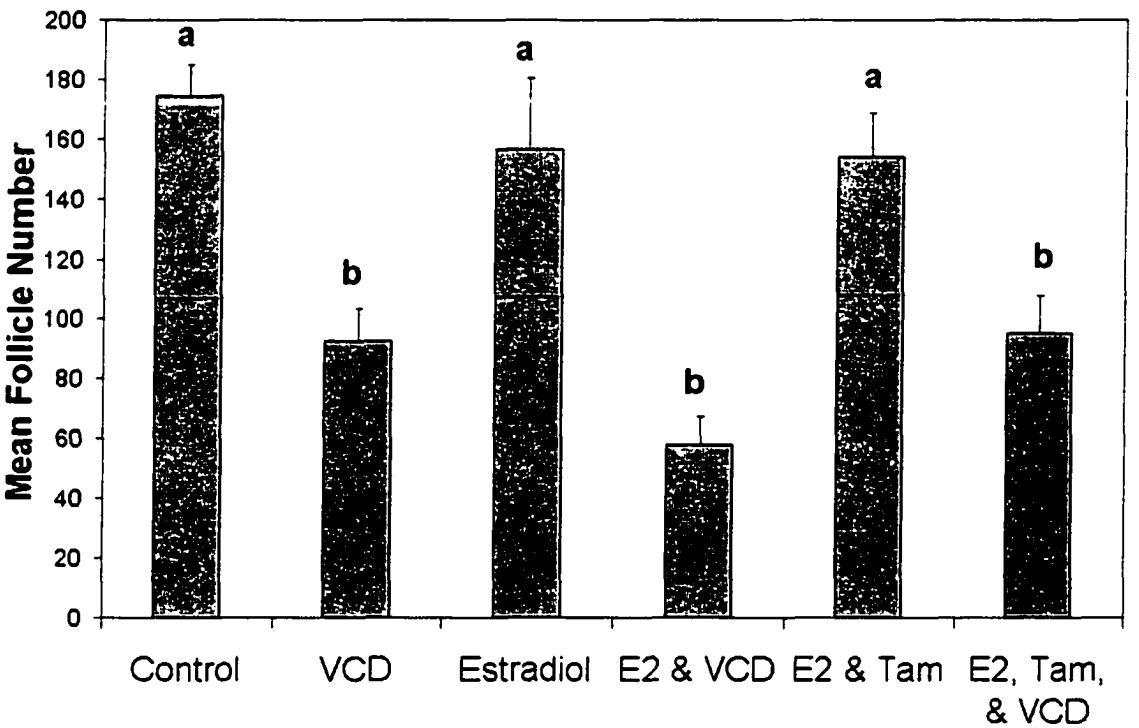


Figure A.6: Effect of dosing with VCD, 17β-estradiol (E_2), and an ER antagonist, 4-hydroxytamoxifen (Tam), on rat primordial follicles. F344 rats (d28) were dosed daily for 15 days with vehicle control (sesame oil), VCD (80 mg/kg, i.p.) ± E_2 (0.1 mg/kg, s.c.), or 4-hydroxytamoxifen (Tam, 2 mg/kg, i.p.), VCD, and E_2. Primordial follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means with common superscripts are statistically the same ($p>0.05$). $N \geq 4$ rats per treatment.

APPENDIX A.2

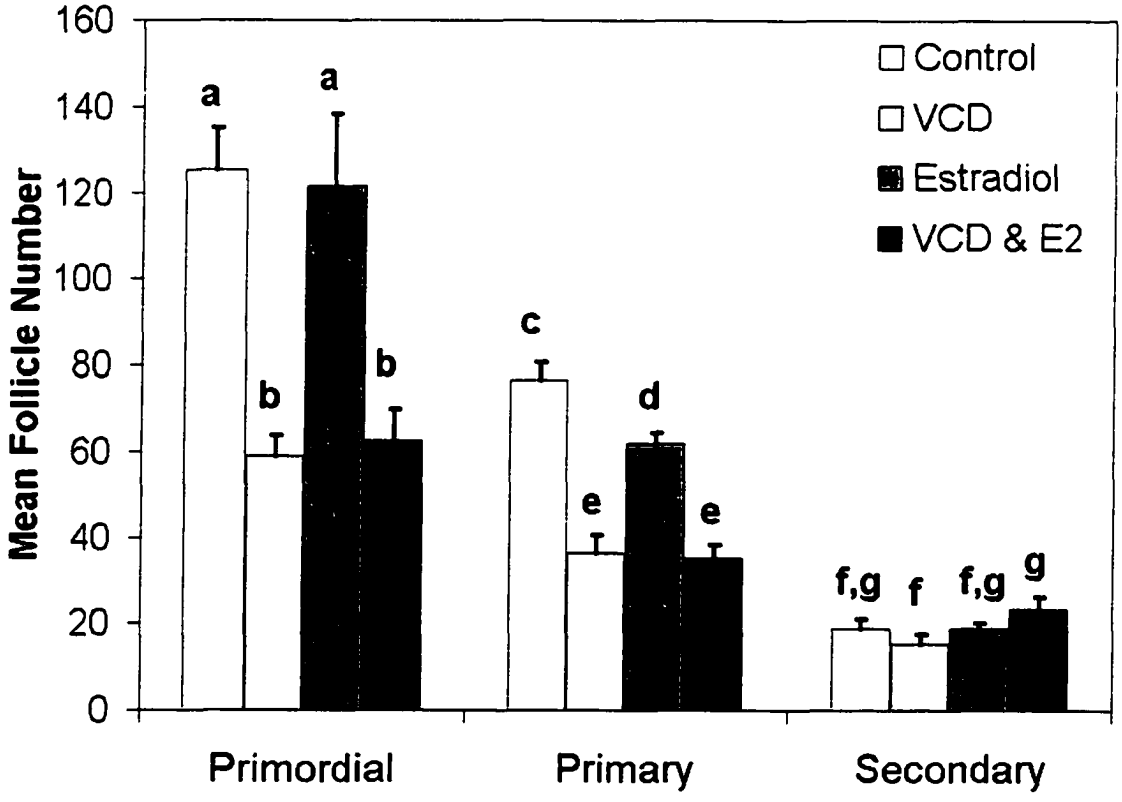


Figure A.7: Effect of VCD and estradiol on mature rat follicles. F344 rats (d60) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), E_2 (0.1 mg/kg, s.c.), or VCD and E_2. Primordial, primary, and secondary follicles were counted in every 40^{th} section after histological staining. Values are mean total follicles counted in each ovary ± standard error; significant was assigned at $p<0.05$. N = 4 rats per treatment.

APPENDIX A.2

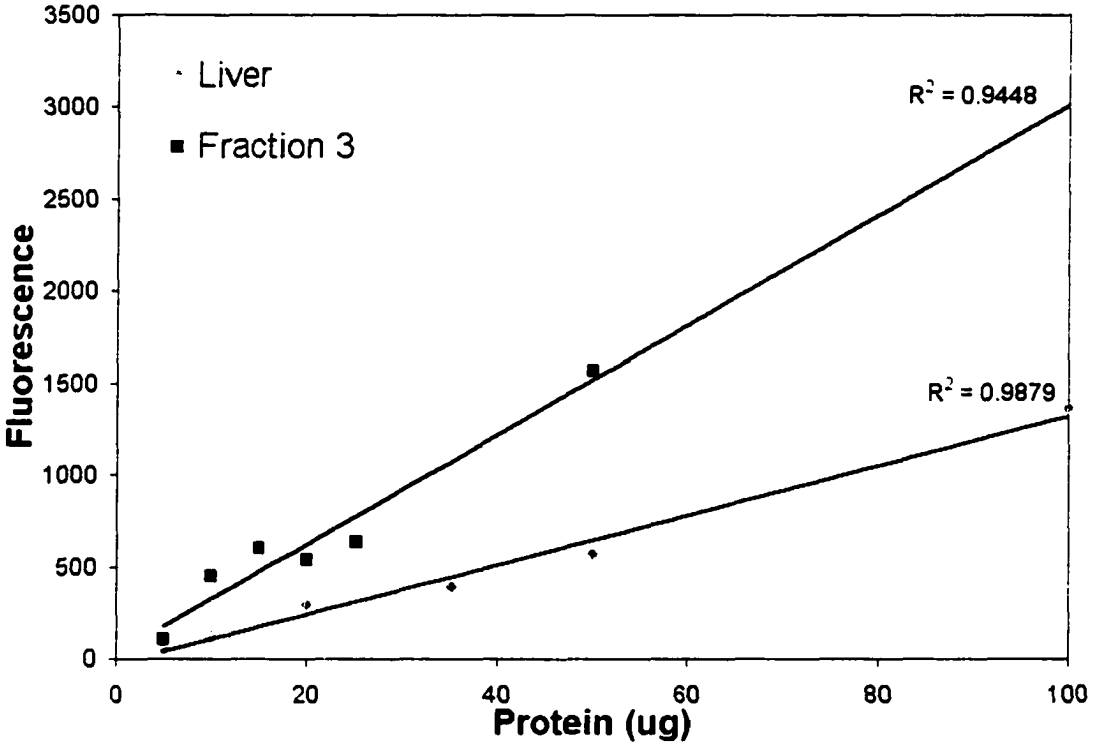
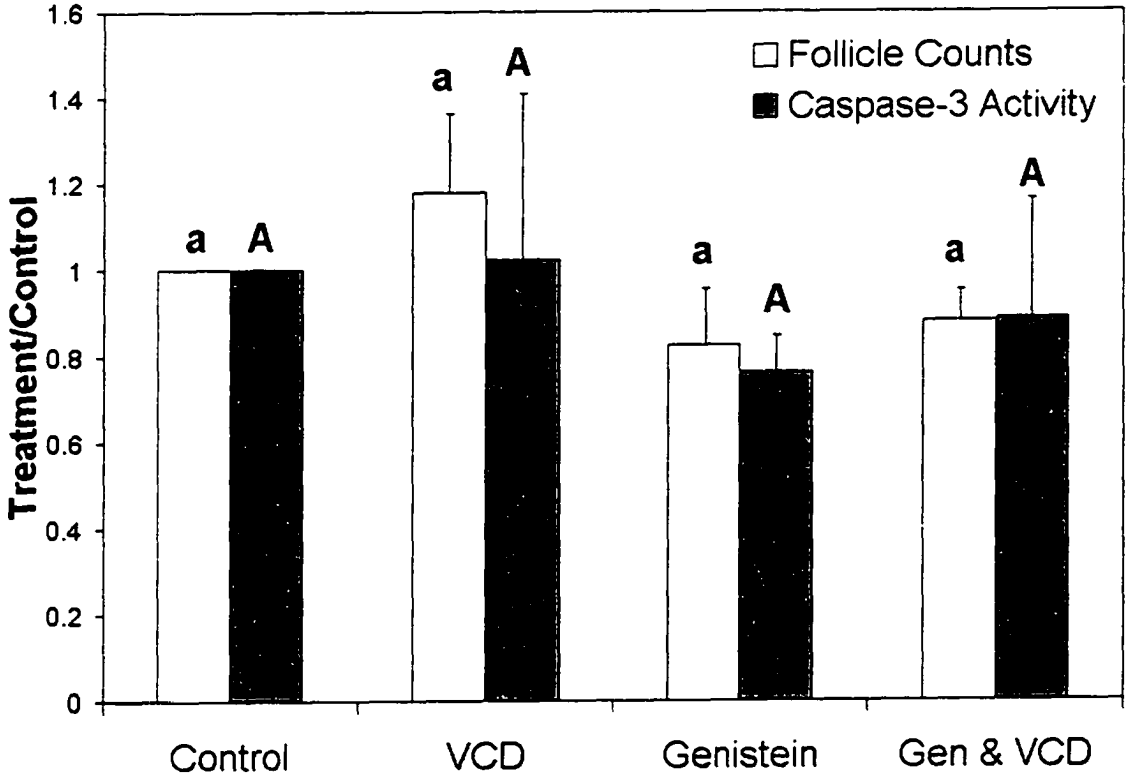


Figure A.8: Caspase-3 activity linearization curve in liver and fraction 3 follicles. Liver or fraction 3 (> 250 μm) follicle cellular homogenate was incubated with a caspase-3 substrate. Production of the cleaved, fluorescent product was made using a fluorescence spectrophotometer. 30 μg protein was selected for future caspase-3 activity experiments as this amount was in the linear range of the assay.

Figure A.9: Effect of dosing with VCD and an ER agonist, genistein (Gen), on rat large pre-antral ovarian follicle numbers and caspase-3-like activity. F344 rats (d28) were dosed daily for 15 days with either vehicle control (sesame oil), VCD (80 mg/kg, i.p.), genistein (0.1 mg/kg, i.p.) or VCD and genistein. Secondary follicles were counted in every 40^{th} section of ovaries as described in materials and methods. Caspase-3-like activity was measured in isolated fraction 2 follicles (100-250 μm) as described in the methods. Open bars; mean follicles counted in each ovary ± standard error ($N \geq 4$ rats per treatment). Shaded bars; mean fluorescence as a measure of caspase-3-like activity ± standard error (N=3); means with similar superscripts are statistically the same ($p>0.05$).

APPENDIX A.2


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Treatment/Control
Follicle Counts
Caspase-3 Activity
a
A
a
A
a
A
a
A
Control
VCD
Genistein
Gen & VCD

APPENDIX A.2

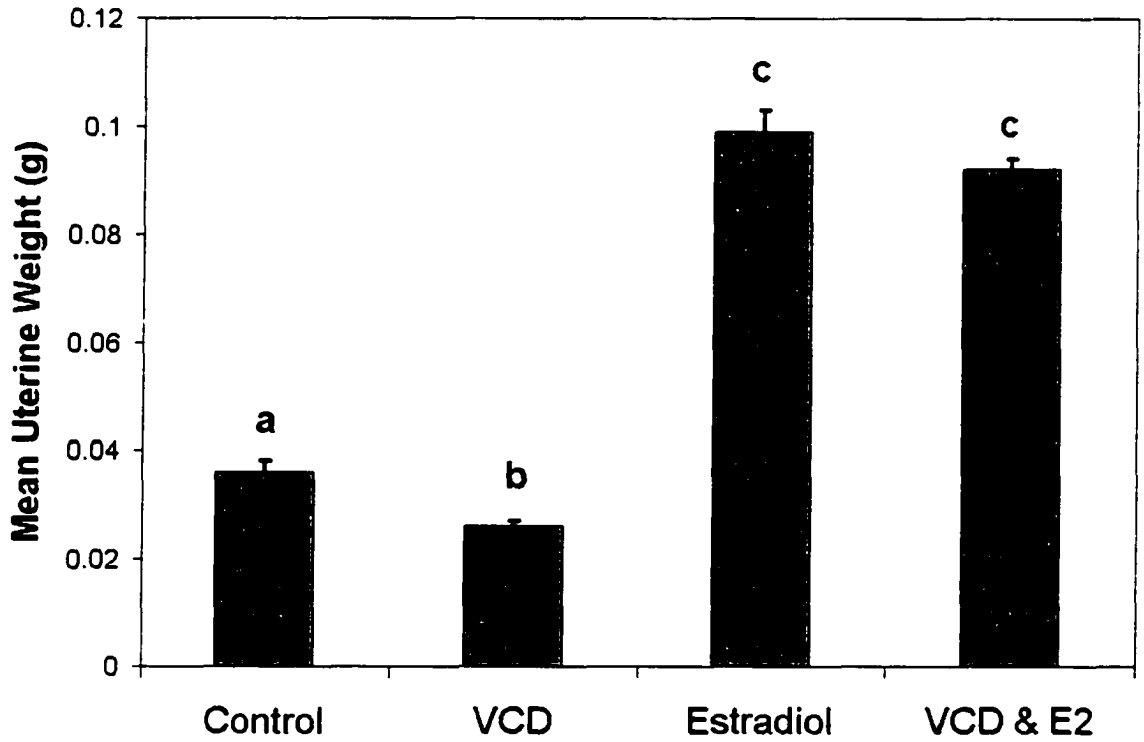


Figure A.10: Effect of VCD on uterine weight of immature rats. F344 rats (d21, N=5-6 rats per treatment) were dosed daily for 3 days with one or more of the following treatments: vehicle control (sesame oil), VCD (80 mg/kg, i.p.), E_2 (0.05 mg/kg, s.c.), or VCD & E_2. Four hours after the final dose, the uterus was removed, trimmed of fat, and weighed. Uterine weight comparisons were made using ANCOVA using body weight as a covariant. Error bars represent standard error and significance was assigned at $p<0.05$.

APPENDIX A.3

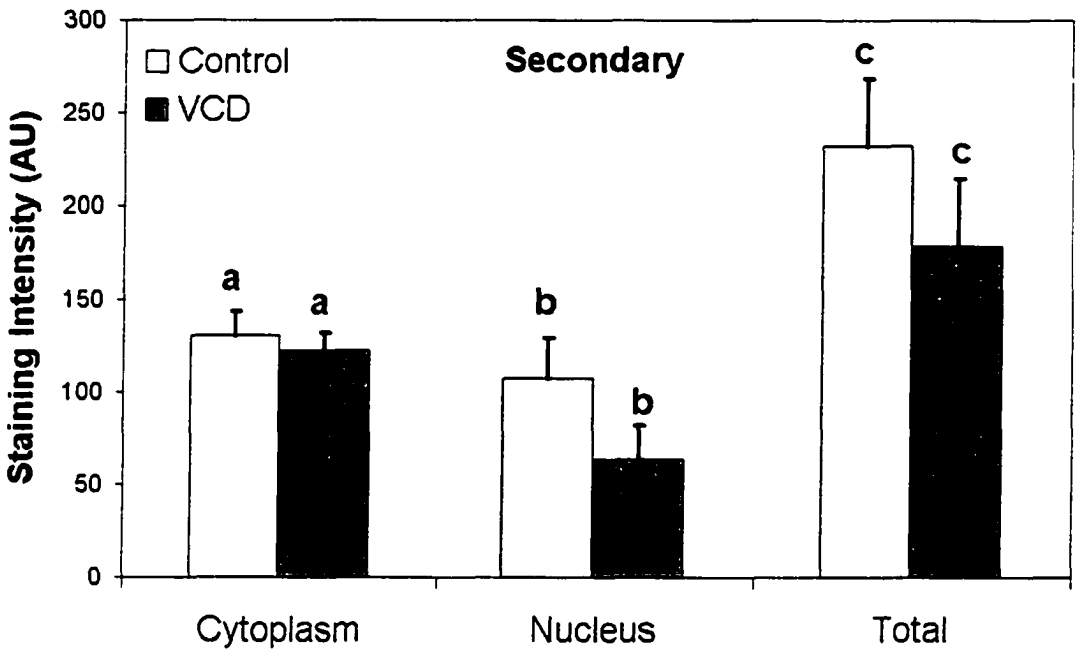


Figure A.11: Staining intensity of Heat Shock Protein 90 (HSP90) in secondary follicles following repeated dosing with VCD in rat ovary. F344 (d28) were treated daily with either vehicle control (sesame oil) or VCD (80 mg/kg, i.p.) for 15 days. Rat ovaries were prepared, stained with a HSP90 antibody, and analyzed with confocal microscopy. Densitometric analysis using Scion Image (NIH) was completed to calculate mean staining in the cytoplasm and nucleus of each follicle in arbitrary units (AU). Bars represent the mean staining for each follicle type ± SE. Means with common superscripts are statistically similar ($p>0.05$).

APPENDIX A.3

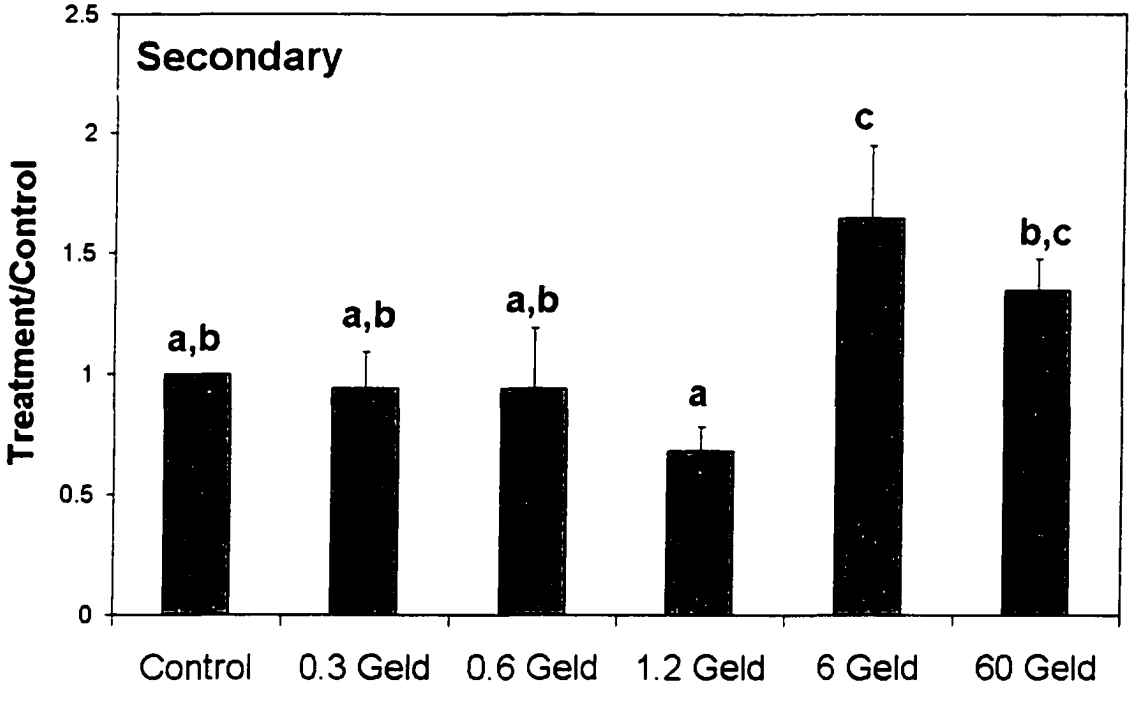


Figure A.12: Effect of geldanamycin on rat secondary follicle numbers. F344 rats (d28) were dosed daily for 15 days with either vehicle control (DMSO, i.p.) or the HSP90 function inhibitor, geldanamycin (Geld, 0.3-60 μg/kg, i.p.). Secondary follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means with common superscripts are statistically the same ($p>0.05$). $N \geq 4$ rats per treatment.

APPENDIX A.3

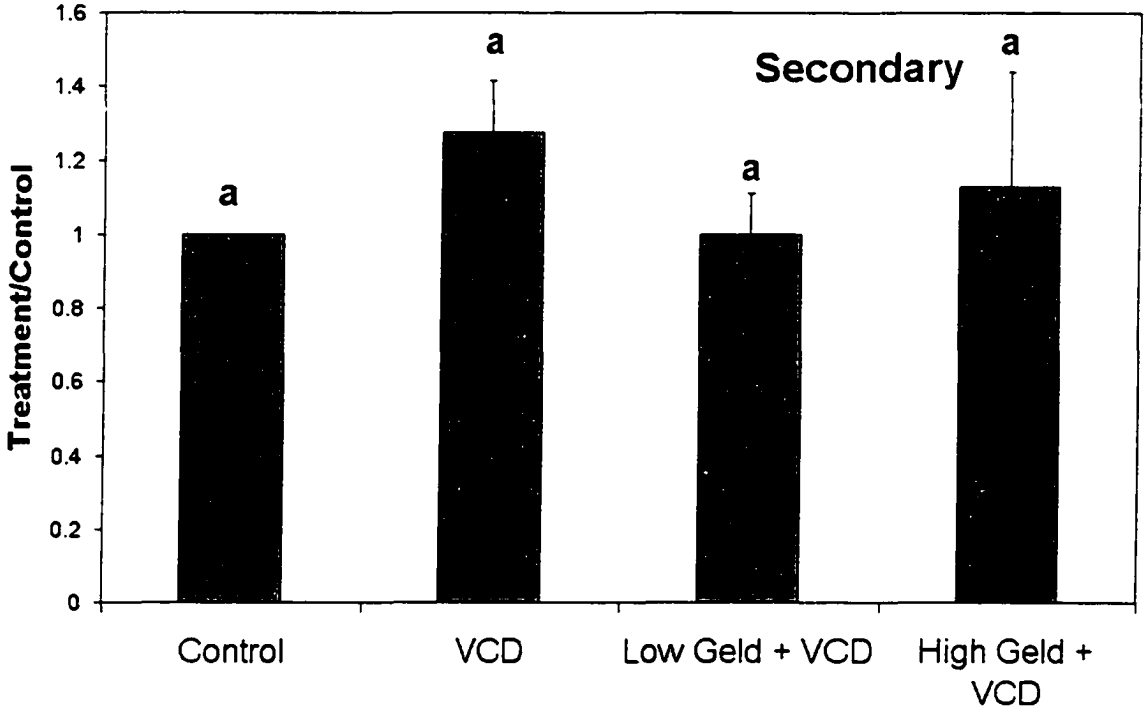


Figure A.13: Effect of VCD and geldanamycin on rat secondary follicle numbers. F344 rats (d28) were dosed daily for 15 days with either vehicle control (DMSO, i.p.), VCD (80 mg/kg, i.p.), or VCD and the HSP90 function inhibitor, geldanamycin (Geld, 6 or 60 μg/kg, i.p.). Secondary follicles were counted in every 40th section as described in Methods. Values are mean total follicles counted in each ovary as a percent of control ± standard error; means with common superscripts are statistically the same ($p>0.05$). $N \geq 4$ rats per treatment.

APPENDIX A.4

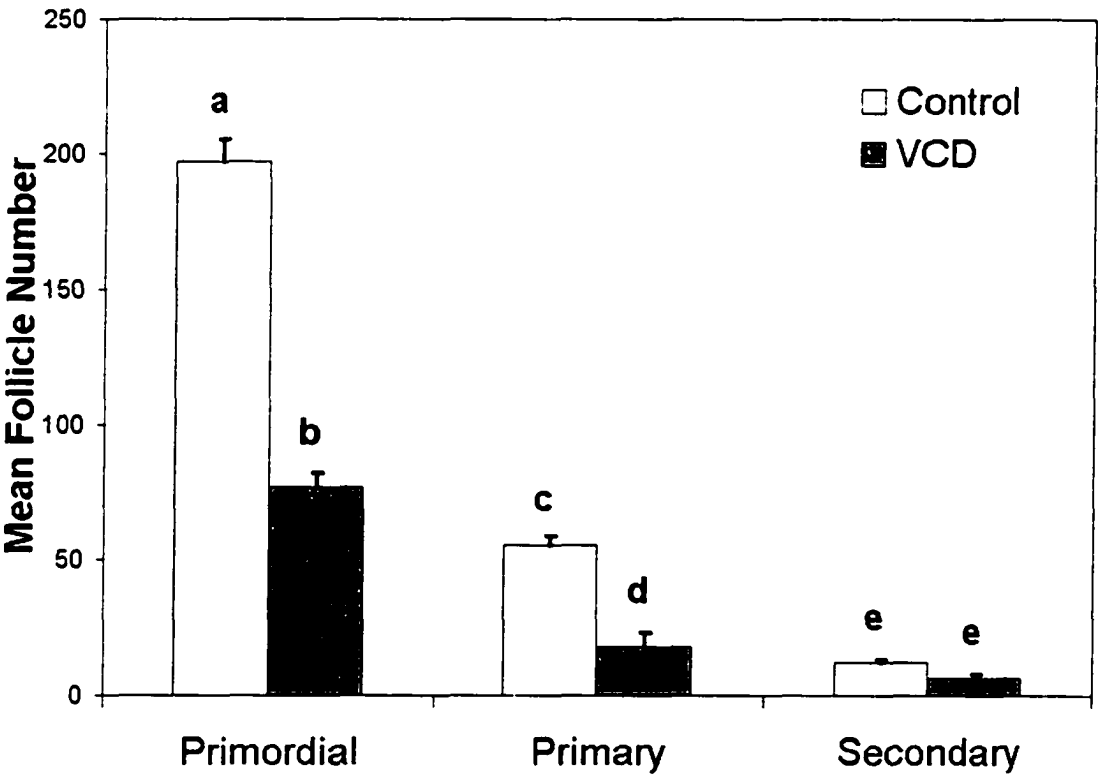


Figure A.14: Effect of dosing with VCD on Sprague-Dawley rats. 28 day old rats were dosed daily for fifteen days with either vehicle control (sesame oil, i.p.) or VCD (80 mg/kg). Four hours after the final dose, ovaries were collected and follicles were counted and classified in every 40th section after histological staining. Values are mean total follicles counted in each ovary ± standard error; means with similar superscripts are statistically the same ($p>0.05$). N = 4-5 rats per treatment.

APPENDIX B

METHODS

REAGENTS

Medium 199 (M199), Trizol, and ϕX 174 RF DNA/HAE III fragments were purchased from Gibco Inc. (Grand Island, NY). VCD (purity > 99%), collagenase (Clostridium histolytium type I), DNase (type I from bovine pancreas), bovine serum albumin, transfer ribonucleic acid (tRNA, type X-SA from Bakers Yeast), estradiol, genistein, 4-hydroxytamoxifen, geldanamycin, alpha-naphthoflavone, sesame oil, and other non-specified reagents were purchased from Sigma (St. Louis, MO). AMV-reverse transcriptase and buffer were purchased from Promega (Madison, WI). Ampli Taq Gold polymerase, PCR buffer, $MgCl_2$, and nucleotides were purchased from Perkin-Elmer (Norwalk, CT). The caspase-3 substrate Ac-DEVD-AMC and the caspase-3 inhibitor (aldehyde) were purchased from Alexis Co. (San Diego, CA). Radiolabeled [α-^{32}P] dCTP (3000 ci/mmol) was purchased from Amersham (Arlington Heights, IL) and [γ-^{32}P] ATP was purchased from Dupont (New England Nuclear).

ANIMALS

Immature female Fischer 344 rats (21 days) were obtained from Harlan Laboratories (Indianapolis, IN) and C57BL/6 mice (21 days) were obtained from Charles

River Laboratories (Wilmington, MA). AhR-deficient mice (AhRKO) mice were originally generated by Schmidt *et al.* (1996). A colony of these mice was established at the University of Maryland School of Medicine Animal Facility and then AhR-deficient (-/-) and heterozygous (+/-) mice were provided as a generous gift from Dr. Jodi A. Flaws. Mice were bred, screened, and genotyped at the University of Maryland as has been previously described (Benedict *et al.*, 2000). Animals were housed in plastic cages, and maintained on 12-h light-dark cycles at a controlled temperature of 22 ± 2°C. Animals were allowed to acclimate to the animal facilities for 1 week prior to initiation of treatment. Rodents were provided food and water *ad libitum*. All experiments were approved by the University of Arizona and University of Maryland Institutional Animal Care and Use Committee and conformed to the Guide for the Care and Use of Experimental Animals.

ANIMAL DOSING

Immature F344 rats or AhR -/-, +/-, and +/+ mice (d28) were weighed and dosed daily for 15 days with one or more treatments (Table C.1). The dose of VCD used has previously been determined to destroy 50% of small preantral follicles (Borman *et al.*, 1999; Springer *et al.*, 1996c). The dose of E_2 used was determined by a dose response study examining follicle counts (Appendix A.2, Figure A.4). The dose of genistein was chosen to be the same as the E_2 dose because genistein has been found to bind ERβ as well as stimulate ER (α & β)-mediated transcription at similar levels as the endogenous

ligand E_2 (Kuiper *et al.*, 1998). The dose of the ER antagonist was selected because 4-hydroxytamoxifen has been shown to inhibit estradiol-induced uterine weight increases at 2.02 mg/kg (Fanidi *et al.*, 1989). The high dose of ANF used was based on a study by Mattison and Thorgeirsson (1979) in which ANF blocked follicle destruction caused by PAHs. Geldanamycin is primarily used *in vitro* to inhibit the activity of HSP90. However, some studies have used this chemical *in vivo* to inhibit protein kinase function and as well as an immunosuppresive agent (Dunlap *et al.*, 1999; Sugita *et al.*, 1999). Subsequently, the initial doses were chosen based on these papers and then a dose response study was completed to examine potential effects in the ovary (Figure 4.4). Four hours after the final dose, animals were euthanized by CO_2 inhalation and ovaries, uterus, liver, and trunk blood were collected.

Table B.1: Details of individual *in vivo* dosing treatments.

Treatment	Abbreviation	Vehicle	Dose	Route
4-Vinylcyclohexene Diepoxide	VCD	Sesame oil or DMSO	80 mg/kg	i.p.
Estradiol	E_2	Sesame oil	0.1 mg/kg	s.c.
Genistein	Gen	Sesame oil	0.1 mg/kg	i.p.
4-Hydroxytamoxifen	Tam	Sesame oil	2 mg/kg	i.p.
α-Naphthoflavone	ANF	Sesame oil	20 mg/kg – low 80 mg/kg - high	i.p.
Geldanamycin	Geld	DMSO	0.3-60 μg/kg	i.p.

RADIOIMMUNOASSAY

Serum 17β-estradiol was measured by radioimmunoassay. Briefly, duplicate serum samples (250 μl) were extracted twice with diethyl ether. Samples were incubated with an E_2 antibody (Sigma; cross reactivity with 17α-estradiol 5%, with estrone 4.8%, with estrone 1.3%, with testosterone and progesterone <0.01%) and ^{3}H-labeled E_2 (NEN; 9 pg/tube) in assay buffer (0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.1% gelatin, 0.1% sodium azide) overnight at 4°C. Samples were then incubated with 200 μl of charcoal solution (0.5% activated charcoal, 0.05% dextran T-70; in assay buffer) for 15 minutes and centrifuged at 6,900 x g for 10 minutes at 4°C. Supernatant was poured into scintillation vials and cpm determined in a scintillation counter (5801 Beckman, Fullerton, CA). The mean sensitivity of the assays was 8.5 pg/tube. The coefficient of variation within the assay was 4.8% and between the assays was 5.8%.

HISTOLOGY AND FOLLICLE COUNTING

Ovaries were trimmed of fat, fixed in Bouin's fixative, embedded, sectioned (5 μm), mounted, and stained with hematoxylin and eosin. In every 40th section (rat) or every 20th section (mouse), pre-antral follicles (containing an oocyte nucleus) were classified as primordial, primary, or secondary and counted as previously described (Pedersen and Peters, 1968; Smith *et al.*, 1990b). For descriptive purposes, primordial

and primary follicles are collectively referred to as small pre-antral follicles. On average, 10.4±0.3 (rat) and 12.8±0.5 (mouse) sections per ovary were evaluated.

FOLLICLE ISOLATION

Ovaries were trimmed of fat, cut into small pieces, and dissociated with BSA, DNase, and collagenase as previously described (Flaws *et al.*, 1994b). Ovarian digests were filtered through a 250 μm screen to exclude large antral follicles (fraction 3, >250 μm). Pre-antral follicles were then hand sorted by size into fraction 1 (25-100 μm, small pre-antral follicles containing primordial, primary and some small secondary follicles) and fraction 2 (100-250 μm, large pre-antral follicles consisting of larger secondary follicles), snap frozen, and stored at -70°C. For each treatment, 12 ovaries from 6 rats were pooled and used as a single observation (N). Each experiment used at least 3 separate groups of animals for each treatment.

RNA PREPARATION

Total RNA was extracted from fraction 1 and 2 follicles by the one-step Trizol method (Chomczynski and Sacchi, 1987). RNA was resuspended in DEPC-treated water and quantified using a spectrophotometer at 260 and 280 nm (DU-64 Beckman, Fullerton, CA).

REVERSE TRANSCRIPTASE-POLYMERASE CHAIN REACTION

0.75 μg RNA was reverse transcribed with AMV-RT as previously described (Borman *et al.*, 1999). 5% of an RT reaction was amplified in the presence of ^{32}P labeled dCTP with primers specific for either rat ERα, ERβ, AhR, GST Ya1, GST Ya2, or 18S (Table C.2; Telleria *et al.*, 1998; Lindros *et al.*, 1998). Conditions of amplification were optimized to be in the linear range of the curve for the AhR, GST Ya1, and GST Ya2. PCR products were separated by 6% PAGE and compared to ϕX 174 RF DNA/Hae III DNA fragments radio-endlabeled with [γ-^{32}P]ATP. Products were verified by oligonucleotide sequencing. The densitometric values obtained for individual PCR signals were normalized to 18S in each sample as described by Camp *et al.* (1991) and Springer *et al.* (1996b). As a control for genomic DNA contamination, reaction tubes were run as described above except no cDNA was added.

Table B.2: Polymerase chain reaction conditions for individual genes.

Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')	Size (Base-pairs)	Conditions (°C, seconds)	Number of Cycles
ERα	AATTCTGACAA TCGACGCCAG	GTGCTTCAACAT TCTCCCTCCTC	344	95°, 60s; 65°, 60s; 72°, 60s	30
ERβ	AAAGCCAAGAG AAACGGTGGGC AT	GCCAATCATGT GCACCAGTTCCT	204	95°, 60s; 65°, 60s; 72°, 60s	30
AhR	CAGGAGTTTCC CTACAAGTCAG AGG	TGTGAGTTTATC CCGTGTCTTTGG	217	95°, 60s; 55°, 60s; 72°, 60s	30
GST Ya1	ATGAGAAGTTT ATACAAAGTCC	GATCTAAAATG CCTTCGGTG	213	95°, 30s; 55°, 60s; 72°, 60s	30
GST Ya2	GATTGACATGT ATTCAGAGGGT	TTTGCATCCATG GCTGGCTT	360	95°, 30s; 57°, 60s; 72°, 60s	35
18S	AACAGCAGCCG CGGTAATTC	TCCCAAGATCC AACTACGAG	80		

IMMUNOFLUORESCENCE AND CONFOCAL MICROSCOPY

Ovaries were fixed in 4% buffered formalin, paraffin embedded, sectioned (5 μm), and deparaffinized. Microwave antigen retrieval in citrate buffer was completed and tissue sections were blocked with 5% BSA/PBS for 5 min. Anti-ERα, anti-ERβ, anti-AhR, or anti-HSP90 (Table C.3) was applied for 1 hr followed by a biotinylated secondary antibody (Vector) at 1:100 dilution (1 hr). Cy5-streptavidin (Jackson Immuno Research Labs) was applied for 1 hr at a 1:50 dilution. Sections were treated with Ribonuclease A (100 μg/ml, Sigma) for 1 hr followed by YOYO-1 (Molecular Probes) staining (5 nM) for 10 min. Slides were repeatedly washed with PBS, cover-slipped with

aqueous mounting medium and stored in the dark at 4°C until viewed on a Leica confocal microscope at 488 and 647 nm with a xenon light source. All incubations were completed at room temperature. Each antibody was verified for specificity and size recognition by western blotting. In each experiment, an immunonegative section was performed for each ovary stained.

Table B.3: Individual antibody conditions for confocal microscopy.

Primary Antibody	Type	Company	Dilution – Primary	Secondary Antibody	Dilution - Secondary
ERα	Rabbit polyclonal	Santa Cruz	1:10	Goat anti-rabbit IgG	1:100
ERβ	Rabbit polyclonal	Upstate Biotechnology	1:20	Goat anti-rabbit IgG	1:100
AhR	Goat polyclonal	Santa Cruz	1:25	Rabbit anti-goat IgG	1:100
HSP90	Goat polyclonal	Santa Cruz	1:25	Rabbit anti-goat IgG	1:100

CONFOCAL MICROSCOPY IMAGE ANALYSIS

Using Scion Image (NIH) software, densitometric analysis of each follicle was assessed by taking several estimates (at least 3) of staining in the cytoplasm and nucleus of each follicle oocyte. For each follicle, all of the measurements were background corrected, averaged, and then added to create an estimate of the total Hsp90 protein in the oocyte. For each follicle type, mean cytoplasmic, nuclear, and total Hsp90 was calculated.

ESTROGEN RECEPTOR ASSAY

Rats (N=6) were dosed daily (i.p.) for 15 days with either vehicle control (sesame oil, i.p.) or VCD (80 mg/kg). For each observation, both ovaries from one animal were homogenized in buffer (0.01 M $NaPO_4$, 0.25 M sucrose, 0.2% sodium azide) supplemented with 0.1 M $MgCl_2$ and 0.1 M β-mercaptoethanol. Homogenates were centrifuged at 100,000g for 1 hr at 4°C and the supernatant was saved as the cytosolic fraction. 60 µl of the cytosolic fraction was assayed for ER number as previously described (Greenstein *et al.*, 1993). Scatchard analysis was used to determine K_d and estrogen receptor number.

ERβ COMPETITOR ASSAY

The ability of VCD to displace an estrogen analog from ERβ was measured as previously described (Bolger *et al.*, 1998) using the PanVera Protocol. Briefly, a fluorescent estrogen (Fluormone™ ES2) was bound to ERβ, various concentrations of VCD were added, and the shift in polarization as the fluorescent estrogen was displaced from the receptor was measured with the Beacon 2000 Fluorescence Polarization Instrument (PanVera Corporation, Madison, WI).

CELLULAR FRACTION ISOLATION

Cytosolic homogenates were isolated from fraction 1 follicles by homogenization in ice-cold lysis buffer (1 % Triton X 100, 50 mM HEPES, pH 7.5, 150 mM NaCl, 10% glycerol, 0.1 % SDS, 2 mM EDTA, 50 mM NaF supplemented with the protease inhibitors PMSF, aprotinin, and leupeptin). Homogenized samples were incubated 30-40 minutes on ice, and centrifuged at 14,000 rpm for 10 minutes. Supernatant was collected and stored at –20°C. Samples of cytosolic fractions were used for protein concentration determination using the enhanced protocol of the BCA Protein assay kit (Pierce, Rockford, IL). Values were measured at 562 nm absorbance with a microplate reader using SOFTmax computer software (Molecular Devices, Sunnyvale, CA).

WESTERN BLOTTING

Fraction 1, 2, or 3 follicle or liver cellular homogenate from d42 control or treated rats was separated by SDS-PAGE and transferred to nitrocellulose. Blots were verified for equal loading of protein across lanes by staining with Ponceau S (0.5%; Sigma). Blots were blocked for 1 hour in 5% dry milk in TTBS (0.5 M NaCl, 20 mM Tris, 0.15% tween-20) and then incubated in primary antibody in 3% dry milk in TTBS for 1 hour at 25°C (Table C.4). Blots were washed three times for 10 minutes each in TTBS and then HRP-conjugated secondary antibody (Santa Cruz, CA) was added for 1 hour at 25°C.

Blots were washed extensively in TBS and then detected with western blotting luminol reagent (Santa Cruz), and exposed to X-ray film.

Table B.4: Conditions for individual antibodies used in western blotting.

Antibody	Type	Company	Protein Quantity (μg)	Dilution - Primary	Secondary	Dilution - Secondary
ERα	Rabbit polyclonal	Santa Cruz	50	1:200	Goat anti-rabbit IgG	1:1000
ERβ	Rabbit polyclonal	Upstate Biotechnology	50	1:200	Goat anti-rabbit IgG	1:1000
Cyp 1A1	Goat polyclonal	Gentest	20 - (follicles) 10 (liver)	1:500	Donkey anti-goat IgG	1:2000
HSP90	Goat polyclonal	Santa Cruz	50	1:200	Donkey anti-goat IgG	1:1000

WESTERN BLOTTING ANALYSIS

Autoradiographs of western blots were evaluated with densitometric analysis. Using an Eagle Eye II system with Eaglesight software version 3.2 (Stratagene, La Jolla, CA), arbitrary densitometric units were assigned to each defined band. An estimate of background was taken from each autoradiograph and subtracted from every individual measurement. Treatment to control ratios were made for each tissue type and compared.

CASPASE-3-LIKE ACTIVITY ASSAY

The cleavage activity of caspase-3 protease was measured as previously described with slight modifications (Hu *et al.*, 2001b). 30 μg of protein was incubated at 37°C for 1 hour in assay buffer (20 mM PIPES, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% (w/v) CHAPS, 10% sucrose, pH 7.2) supplemented with 50 M of the caspase-3 substrate Ac-DEVD-AMC. Substrate cleavage was detected by measurement of the fluorescence of free 7-amino-4-methylcoumarin (AMC) with a F-2000 fluorescence spectrophotometer (Hitachi, Ltd, Tokyo, Japan) using excitation at 380 nm and detection emission at 460 nm. This assay was verified by use of a caspase-3 inhibitor (aldehyde).

STATISICAL ANALYSIS

For each experiment, the data ($N \geq 3$) were averaged for each treatment. For the caspase-3-like activity, RT-PCR, and ER antagonist studies, treatment/control ratios were calculated and analyzed. Data are presented as means (or mean ratios) ± SE. Comparisons between all groups were made using one-way ANOVA and Fisher's PLSD (Protected Least Significant Difference) analysis. Data were checked for normal distribution with the Shapiro-Wilk W Test and verified for heterogeneity of variance with the F_{max} Test. Significance was assigned at $p<0.05$. Means that share common superscripts are statistically similar ($p>0.05$).

REFERENCES

Abbott, DB, Schmid, JE, Pitt, JA, Buckalew, AR, Wood, CR, Held, GA, and Diliberto, JJ. 1999. Adverse reproductive outcomes in the transgenic Ah receptor-deficient mouse. *Toxicology and Applied Pharmacology*. 155(1): 62-70.

Ahluwalia A, Burke KA, Osborne EZ, Gize EA, Bigsby RM, Pope WF, and Nephew KP. 1998. Estrogen receptor-β mRNA expression in the sheep ovary. In: Program of the 31st annual meeting of the Society for Study of Reproduction; 1998; College Station, Texas. Abstract 124.

Baird, DD and Wilcox, AJ. 1985. Cigarette smoking associated with delayed conception. *JAMA*. 253: 2979-83.

Baker, TG. 1963. A quantitative and cytological study of germ cells in human ovaries. *Proceedings of the Royal Society of London [Biology]*. 158: 417-33.

Balmer, C and Valley, AW. 1997. *Basic principles of cancer treatment and cancer chemotherapy, in Pharmacotherapy: A Pathophysiologic Approach* 3rd Edition. (DiPiro, JT, Talbert, RL, Yee, GC, Matzke, GR, Wells, BG, and Posey, LM, eds), pp. 2434. Appleton and Lange, Stanford, CT.

Basset, DL. 1943. The changes in the vascular pattern of the ovary of the albino rat during the estrous cycle. *American Journal of Anatomy*. 73: 251-291.

Benedict, JC, Lin, T-M, Loeffler, IK, Peterson, RE, and Flaws, JA. 2000. Physiological role of the aryl hydrocarbon receptor in mouse ovary development. *Toxicological Sciences*. 56: 382-388.

Benedict, JC, Lin, T-M, Peterson, RE, and Flaws, JA. 2001. The aryl hydrocarbon receptor (AhR) may regulate the growth of ovarian follicles to the antral stage. *Biology of Reproduction*. 64 (Supplement 1): Abstract 12.

Berbers, GAM, Kunnen, R, Van Bergen en Henegouwen, PMP, and Van Wijk, R. 1988. Localization and quantitation of hsp84 in mammalian cells. *Experimental Cell Research*. 177: 257-271.

Billig, H, Furuta, I, and Hsueh, AJW. 1993. Estrogens inhibit and androgens enhance ovarian granulosa cell apoptosis. *Endocrinology*. 133(5): 2204-2212.

Block, E. 1952. Quantitative morphological investigations of the follicular system in women. Variations at different ages. *Acta Anatomica*. 14: 108-23.

Bokemeyer, C, Schmoll, HJ, vanRhee, J, Kuczyk, M, Schuppert, F, and Poliwoda, H. 1994. Long-term gonadal toxicity after therapy for Hodgkin's and non-Hodgkin's lymphoma. *Annals of Hematology*. 68(3): 105-110.

Bolger, R, Wiese, TE, Ervin, K, Nestich, S, Checovich, W. 1998. Rapid screening of environmental chemicals for estrogen receptor binding capacity. *Environmental Health Perspectives*. 106: 551-557.

Bolon, B, Bucci, TJ, Warbritton, AR, Chen, JJ, Mattison, DR, and Heindel, JJ. 1997. Differential follicle counts as a screen for chemically induced ovarian toxicity in mice: results from continuous breeding bioassays. *Fundamental and Applied Toxicology*. 39: 1-10.

Borman, S.M., VanDePol, B.J., Kao, S., Thompson, K.E., Sipes, I.G., and Hoyer, P.B. 1999. A single dose of the ovotoxicant 4-vinylcyclohexene diepoxide is protective in rat primary follicles. *Toxicology and Applied Pharmacology*. 158: 244-252.

Borman, SM, Christian, PJ, Sipes, IG, and Hoyer, PB. 2000. Ovotoxicity in female Fischer rats and B6 mice induced by low dose exposure to three polycyclic aromatic hydrocarbons: Comparison through calculation of an ovotoxic index (OI). *Toxicology and Applied Pharmacology*. 167: 191-198.

Brinton, LA, Barrett, RJ, Berman, ML, Mortel, R, Twiggs, LB, and Wilbanks, GD. 1993. Cigarette smoking and the risk of endometrial cancer. *American Journal of Epidemiology*. 137(3): 281-91.

Buchner, J. 1999. Hsp90 & Co.- a holding for folding. *Trends in Biochemical Sciences*. 24(4): 136-41.

Burbach, KM, Poland, A, and Bradfield, CA. 1992. Cloning of the Ah-receptor cDNA reveals a distinctive ligand-activated transcription factor. *Proceedings of the National Academy of Sciences of the USA*. 89: 8185-89.

Byskov, AG. 1986. Differentiation of mammalian embryonic gonad. *Physiology Reviews*. 66: 71-117.

Cai, J., Yang, J., and Jones, D.P. 1998. Mitochondrial control of apoptosis: the role of cytochrome *c*. *Biochimica et Biophysica Acta*. 1366(1-2): 139-49.

Camp, TA, Rahal, JO, and Mayo, KE. 1991. Cellular localization and hormonal regulation of follicle-stimulating hormone and luteinizing hormone receptor messenger RNA's in the rat ovary. *Molecular Endocrinology*. 5: 1405-1417.

Caplan, AJ. 1999. HSP90's secrets unfold: new insights from structural and functional studies. *Trends in Cell Biology*. 9: 262-8.

Carandang, JM, Franco-Bronson, K, and Kamarei, S. 2000. Recognizing and managing depression in women throughout the stages of life. *Cleveland Clinic Journal of Medicine*. 67(5): 329-31, 335-338.

Carlsten, H, Nilsson, N, Jonsson, R, Backman, K, Holmdahl, R, and Tarkowsky, A. 1992. Estrogen accelerates immune complex glomerulonephritis but ameliorates T-cell mediated vasculitis and sialadentitis in autoimmune MRL lpr/lpr mice. *Cellular Immunology*. 144: 190-202.

Carlsten, H and Tarkowsky, A. 1993. Histocompatibility complex gene production and exposure to oestrogens: two independent disease acceleration functions in murine lupus. *Scandinavian Journal of Immunology*. 38: 341-347.

Carson-Jurica, M.A., Schrader, W.T., O'Malley, B.W. 1990. Steroid receptor family: structure and functions. *Endocrine Reviews*. 11(2): 201-220.

Caruso, JA, Laird, DW, and Batist, G. 1999. Role of HSP90 in mediating cross-talk between the estrogen receptor and the Ah receptor signal transduction pathways. *Biochemical Pharmacology*. 58: 1395-1403.

Carver, LA, Jackiw, V, and Bradfield, CA. 1994. The 90-kDa heat shock protein is essential for Ah receptor signaling in a yeast expression system. *Journal of Biological Chemistry*. 269: 30109-12.

Carver, LA, LaPres, JJ, Jain, S, Dunham, EE, and Bradfield, CA. 1998. Characterization of the Ah receptor-associated protein, APA9. *Journal of Biological Chemistry*. 273(50): 33580-7.

Cattanach, B.M. 1959. The effect of triethylene-melamine on the fertility of female mice. *International Journal of Radiation Biology*. 3: 288-92.

Chambraud, B, Berry, M, Redeuilh, G, Chambon, P, and Baulieu, EE. 1990. Several regions of human estrogen receptor are involved in the formation of receptor-heat shock protein 90 complexes. *Journal of Biological Chemistry*. 265: 20686-91.

Chang, TK, Gonzalez, FJ, and Waxman, DJ. 1994. Evaluation of triacetyloleandomycin, alpha-naphthoflavone and diethyldithiocarbamate as selective chemical probes for inhibition of human cytochromes P450. *Archives of Biochemistry and Biophysics*. 311: 437-442.

Channing, CP, Schaerf, FW, Anderson, LD, and Tsafriri A. 1980. Ovarian follicular and luteal physiology. *International Review of Physiology*. 22: 117-201.

Chun, S-Y, Billig, H, Tilly, JL, Furuta, I, Tsafriri, A, and Hsueh, AJW. 1994. Gonadotropin suppression of apoptosis in cultured preovulatory follicles: mediatory role of endogenous insulin-like growth factor I. *Endocrinology*. 135: 1845-1853.

Chun, S-Y, Eisenhauer, KM, Kubo, M, and Hsueh, AJW. 1995. Interleukin-1β suppresses apoptosis in rat ovarian follicles by increasing nitric oxide production. *Endocrinology*. 136(7): 3120-3127.

Chun, S-Y and Hsueh, AJW. 1998. Paracrine mechanisms of ovarian follicle apoptosis. *Journal of Reproductive Immunology*. 39: 63-75.

Colella, TA and Gallo, MA. 1993. *The Toxicologist*. 13: 102.

Chomczynski, P and Sacchi, N. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry*. 162: 156-159.

Coumailleau, P, Poellinger, L, Gustafsson, JA, and Whitelaw, ML. 1995. Definition of a minimal domain of the dioxin receptor that is associated with Hsp90 and maintains wild type ligand binding affinity and specificity. *Journal of Biological Chemistry*. 270: 25291-300.

Cowan, KH, Batist, G, Tulpule, A, Sinha, BK, and Myers, CE. 1986. Similar biochemical changes associated with multidrug resistance in human breast cancer cells and carcinogen-induced resistance to xenobiotics in rats. *Proceedings of the National Academy of Sciences of the USA*. 83: 9328-32.

Cutson, TM and Meuleman, E. 2000. Managing menopause. *American Family Physician*. 61(5): 1405-1406.

Davis, BJ, Weaver, R, Gaines, LJ, and Heindel, JJ. 1994. Mono-(2-ethylhexyl) phthalate suppresses estradiol production independent of FSH-cAMP stimulation in rat granulosa cells. *Toxicology & Applied Pharmacology*. 128(2): 224-8.

Denison, MS, Phelan, D, and Elferink, CJ. 1998. In: *Toxicant-Receptor Interactions*. (Denison, MS and Helferich, WG, Eds.), pp. 3-33, Taylor and Francis, Philadelphia.

Denison, MS and Heath-Pagliuso, S. 1998. The Ah Receptor: a regulator of the biochemical and toxicological actions of structurally diverse chemicals. *Bulletin of Environmental Contamination and Toxicology*. 61: 557-568.

Devine, PJ, Sipes, IG, and Hoyer, PB. 2001. Effect of 4-vinylcyclohexene diepoxide dosing in rats on GSH levels in liver and ovaries. *Toxicological Sciences*. 62: 315-320.

DeVito, M, Thomas, T, Martin, E, Umbreit, T, and Gallo, M. 1990. *The Toxicologist*. 10: (246).

Devito, MS and Birnbaum, LS. 1994. Toxicology of dioxins and related chemicals. In: A. Schecter, (ed) *Dioxins and Health*, Plenum Press, New York p 139.

Doerr, JK, Hollis, EA, and Sipes, IG. 1996. Species differences in the ovarian toxicity of 1,3-butadiene epoxides in B6C3F$_1$ mice and sprague-dawley rats. *Toxicology*. 113: 128-136.

Driancourt, MA, Guet, P, Reynaud, K, Chadli, A, and Catelli, MG. 1999. Presence of an aromatase inhibitor, possibly heat shock protein 90, in dominant follicles of cattle. *Journal of Reproduction and Fertility*. 115: 45-58.

Duan, R, Porter, W, Samudio, I, Vyhlidal, C, Kladde, M, and Safe, S. 1999. Transcriptional activation of c-fos protooncogene by 17β-estradiol: mechanism of aryl hydrocarbon receptor-mediated inhibition. *Molecular Endocrinology*. 13(9): 1511-1521.

Dunlap, DY, Moreno-Aliaga, MJ, Wu, Z, and Matsumura, F. 1999. Differential toxicities of TCDD in vivo among normal, c-src knockout, geldanamycin- and quercetin-treated mice. *Toxicology*. 135: 95-107.

Ekena, K, Weis, KE, Katzenellenbogen, JA, and Katzenellenbogen, BS. 1997. Different residues of the human estrogen receptor are involved in the recognition of structurally diverse estrogens and antiestrogens. *Journal of Biological Chemistry*. 272: 5069-5075.

Ema, M., Sogawa, K., Watanabe, N., Chujoh, Y., Matsushita, N., Gotoh, O., Funae, Y., and Fujii-Kuriyama, Y. 1992. cDNA cloning and structure of the mouse putative Ah receptor. *Biochemical and Biophysical Research Communications*. 184: 246-53.

Enan, E, Lasley, B, Stewart, D, Overstreet, J, and Vandervoort, CA. 1996. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) modulates function of human luteinizing granulosa cells via cAMP signaling and early reduction of glucose transporting activity. *Reproductive Toxicology*. 10: 191-8.

Enmark, E., Pelto-Huikko, M., Grandien, K., Lagercrantz, S., Lagercrantz, J., Fried, G., Nordenskjold, M., and Gustafsson, J-A. 1997. Human estrogen receptor β-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism*. 1997. 82(12): 4258-4265.

Enmark, E. and Gustafsson, J-A. 1999. Oestrogen receptors-an overview. *Journal of Internal Medicine.* 246: 133-138.

Erickson, GF. 1978. Normal Ovarian Function. *Clinical Obstetrics and Gynecology.* 21: 31-52.

Eshkol, A and Lunenfeld, B. 1972. Gonadotropic regulation of ovarian development in mice during infancy. In Saxena, BB, Gandy, HM, Beling, CG (eds): *Gonadotropins.* New York, Wiley.

Evans, M.J., McLaughlin, S., Marvin, R.D., and Abdou, N.I. 1997. Estrogen decreases *in vitro* apoptosis of peripheral blood mononuclear cells from women with normal menstrual cycles and decreases TNF-α production in SLE but not in normal cultures. *Clinical Immunology and Immunopathology.* 82(3): 258-262.

Familiari, G., Caggiati, A, Nottola, SA, Ermini, M, DiBenedetto, MR, and Motto, PM. 1993. Ultrastructure of human ovarian primordial follicles after combination chemotherapy for Hodgkin's disease. *Human Reproduction.* 8(12): 2080-2087.

Fanidi, A, Courion-Guichardaz, C, Fayard, JM, Pageaux, JF, and Laugier, C. 1989. Effects of tamoxifen, tamoxifen metabolites, and nafoxidine on adenosine 3',5'-monophosphate phosphodiesterase: correlations with growth inhibitory activities but not estrogen receptor affinities. *Endocrinology.* 125: 1187-1193.

Fernandez-Salguero, P, Pineau, T, Hilbert, DM, McPhail, T, Lee, SS, Kimura, S, Nebert, DW, Rudikoff, S, Ward, JM, and Gonzalez, FJ. 1995. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. *Science.* 268(5211): 722-726.

Fisher, C.R., Graves, K.H., Parlow, A.F., and Simpson, E.R. 1998. Characterization of mice deficient in aromatase (ArKO) because of targeted disruption of the *cyp19* gene. *Proceedings of the National Academy of Sciences.* 95: 6965-6970.

Flaws, JA, Doerr, JK, Sipes, IG, and Hoyer, PB. 1994a. Destruction of preantral follicles in adult rats by 4-vinyl-1-cyclohexene diepoxide. *Reproductive Toxicology.* 8(6): 509-514.

Flaws, JA, Salyers, KL, Sipes, IG, and Hoyer, PB. 1994b. Reduced ability of rat preantral ovarian follicles to metabolize 4-vinyl-1-cyclohexene diepoxide *in vitro. Toxicology and Applied Pharmacology.* 126: 286-294.

Flaws, JA, and Sommer, RJ, Silbergeld, EK, Peterson, RE, and Hirshfield, AN. 1997. *In utero* and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) induces

genital dysmorphogenesis in the female rat. *Toxicology and Applied Pharmacology.* 147: 351-362.

Franc, MA, Pohjanvirta, R, Tuomisto, J, and Okey, AB. 2001. Persistent, low-dose 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure: effect on aryl hydrocarbon receptor expression in a dioxin-resistance model. *Toxicology and Applied Pharmacology.* 175: 43-53.

Franceschi, S, Gallus, S, Talamini, R, Tavani, A, Negri, E, and La Vecchia, C. 2000. Menopause and colorectal cancer. *British Journal of Cancer.* 82(11): 1860-1862.

Garcia-Segura, LM, Cardona-Gomez, P, Naftolin, F, and Chowen, JA. 1998. Estradiol upregulates bcl-2 expression in adult brain neurons. *Neuroreport.* 9(4): 593-597.

Garnier, M, Di Lorenzo, D, Albertini, A, and Maggi, A. 1997. Identification of estrogen-responsive genes in neuroblastoma SK-ER3 cells. *Journal of Neuroscience.* 17: 4591-4599.

Gierthy, JF, Lincoln, DW, Gillespie, MB, Seeger, JI, Martinez, HL, Dickerman, HW, and Kumar, SA. 1987. Suppression of estrogen-regulated extracellular tissue plasminogen activator activity of MCF-7 cells by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Cancer Research.* 47: 6198-6203.

Gillesby, B, Santostefano, M, Porter, W, Wu, ZF, Safe, S, and Zacharewxki, T. 1997. Identification of a motif within the 5' regulatory region of pS2 which is responsible for AP-1 binding and TCDD-mediated suppression. *Biochemistry.* 36(20): 6080-9.

Gollapudi, L and Oblinger, MM. 1999a. Stable transfection of PC12 cells with estrogen receptor (ERα): protective effects of estrogen on cell survival after serum deprivation. *Journal of Neuroscience Research.* 56: 99-108.

Gollapudi, L and Oblinger, MM. 1999b. Estrogen and NGF synergistically protect terminally differentiated, ERα-transfected PC12 cells from apoptosis. *Journal of Neuroscience Research.* 56: 471-481.

Gondos, B. 1970. Germ cell relationships in the developing rabbit ovary, in Gonadotropins and ovarian development. (Butt, WR, Crooke, AC, and Ryle, M, eds), pp. 239-247. Livingstone, Edinburgh.

Gonzalez, FJ and Nebert, DW. 1985. Autoregulation plus upstream positive and negative control regions associated with transcriptional activation of the mouse $P_1$450 gene. *Nucleic Acids Research.* 13: 7269-7288.

Gonzalez, FJ, Fernandez-Salguero, P, Lee, SST, Pineau, T, and Ward, JM. 1995. Xenobiotic receptor knockout mice. *Toxicology Letters*. 82/83: 117-121.

Gonzalez, FJ and Fernandez-Salguero, P. 1998. The aryl hydrocarbon receptor: studies using the AHR-null mice. *Drug Metabolism and Disposition*. 26: 1194-1198.

Goodman, S.B., Kugu, K., Chen, S.H., Preutthipan, S., Tilly, K.I., Tilly, J.L., and Dharmarajan, A.M. 1998. Estradiol-mediated suppression of apoptosis in the rabbit corpus luteum is associated with a shift in expression of bcl-2 family members favoring cellular survival. *Biology of Reproduction*. 59(4): 820-7.

Gosden, RG. 1987. Follicular status at the menopause. *Human Reproduction*. 2(7): 617-621.

Gray, RH. 1976. The menopause-epidemiological and demographic considerations. In Beard, R.J. (ed), *The Menopause*. MTP Press, Lancaster, pp. 25-39.

Gray, LE and Ostby, JS. 1995. *In utero* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) alters reproductive morphology and function in female rat offspring. *Toxicology and Applied Pharmacology*. 133: 285-294.

Green, S., Walter, P., Kumar, V., Krust, A, Bornert, JM, Argos, P, Chambon, P. 1986. Human oestrogen receptor cDNA: sequence, expression, and homology to v-erbA. *Nature*. 320: 134-9.

Greenstein, BD, Dhaher, YY, Bridges, E de F, Keser, G, Khamashta, MA, Etherington, J, Greenstein, AS, Coates, PJ, Hall, PA, and Hughes, GR. 1993. Effects of an aromatase inhibitor on thymus and kidney and on oestrogen receptors in female MRL/MP-lpr/lpr mice. *Lupus*. 2: 221-225.

Grizzle, TB, George, JD, Fail, PA, Seely, JC, and Heindel, JJ. 1994. Reproductive effects of 4-vinylcyclohexene in swiss mice assessed by a continuous breeding protocol. *Fundamentals of Applied Toxicology*. 22: 122-129.

Gross, A, McDonnell, JM, and Korsmeyer, SJ. 1999. BCL-2 family members and the mitochondria in apoptosis. *Genes & Development*. 13(15): 1899-911.

Gu, Y-Z, Hogenesch, JB, and Bradfield, CA. 2000. The PAS superfamily: sensors of environmental and developmental signals. *Annual Review of Pharmacology and Toxicology*. 40: 519-61.

Guraya, SS. 1985. *Biology of Ovarian Follicles*. Springer-Verlag, New York.

Gustafsson, J-A. 1999. Estrogen receptor β - a new dimension in estrogen mechanism of action. *Journal of Endocrinology*. 163: 379-383.

Haggin, J. 1994. New styrene process uses crude butadiene. *Chemical and Engineering News*. 72(25): 31-32.

Hahn, ME. 1998. The aryl hydrocarbon receptor: a comparative perspective. *Comparative Biochemistry and Physiology*. Part C 121: 23-53.

Hankinson, O. 1995. The aryl hydrocarbon receptor complex. *Annual Review of Pharmacology and Toxicology*. 35: 307-340.

Healy, C, Milmore, JE, and Guideri, G. 1991. Effects ot ethinyl estradiol on isoproterenol-induced death in ventricular fibrillation in DOCA-salt pretreated male and female rats. *Life Sciences*. 48: 1931-6.

Heimler, I, Rawlins, RG, Owen, H, and Hutz, RJ. 1998a. Dioxin perturbs, in a dose- and time-dependent fashion, steroid secretion, and induces apoptosis of human luteinized granulosa cells. *Endocrinology*. 139(10): 4373-9.

Heimler, I, Trewin, AL, Chaffin, CL, Rawlins, RG, and Hutz, RJ. 1998b. Modulation of ovarian follicle maturation and effects on apoptotic cell death in Holtzman rats exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) *in utero* and lactationally. *Reproductive Toxicology*. 12: 69-73.

Hirshfield, AN. 1991a. Development of follicles in the mammalian ovary. *International Review of Cytology*. 124: 43-100.

Hirshfield, AN. 1991b. Theca cells may be present at the onset of follicular growth. *Biology of Reproduction*. 44: 1157-6.

Hirshfield, A.N. 1997. Overview of ovarian follicular development: considerations for the toxicologist. *Environmental and Molecular Mutagenesis*. 29: 10-15.

Hoffman, EC, Reyes, H, Chu, FF, Sander, F, Conley, LH, Brooks, BA, Hankinson, O. 1991. Cloning of a factor required for activity of the Ah (dioxin) receptor. *Science*. 252: 954-958.

Hooser, SB, Parola, LR, Van Ert, MD, and Sipes, IG. 1993. Differential ovotoxicity of 4-vinylcyclohexene and its analog, 4-phenylcyclohexene. *Toxicology and Applied Pharmacology*. 119: 302-305.

Hooser, SB, Douds, DP, DeMerell, DG, Hoyer, PB, and Sipes, IG. 1994. Long-term ovarian and gonadotropin changes in mice exposed to 4-vinylcyclohexene. *Reproductive Toxicology*. 8: 315-323.

Hostein, I, Robertson, D, DiStefano, F, Workman, P, and Clarke, PA. 2001. Inhibition of signal transduction by the Hsp90 inhibitor 17-allyamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. *Cancer Research*. 61(10): 4003-4009.

Howell, S and Shalet, S. 1998. Gonadal damage from chemotherapy and radiotherapy. *Endocrinology & Metabolism Clinics of North America*. 27(4): 927-943.

Hoyer, PB and Sipes, IG. 1996. Assessment of follicle destruction in chemical-induced ovarian toxicity. *Annual Review of Pharmacology and Toxicology*. 36: 307-331.

Hruska, RE and Olson, JR. 1989. Species differences in estrogen receptors and in the response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin exposure. *Toxicology Letters*. 48(3): 289-299.

Hsu, SY and Hsueh, AJ. 2000. Tissue-specific bcl-2 protein partners in apoptosis: an ovarian paradigm. *Physiological Reviews*. 80(2): 593-614.

Hsueh, AJW, Billig, H, and Tsafriri, A. 1994. Ovarian follicle atresia: a hormonally controlled apoptotic process. *Endocrine Reviews*. 15(6): 707-724.

Hu, X, Christian, PJ, Sipes, IG, and Hoyer, PB. 2001a. Expression and Redistribution of Cellular Bad, Bax, and Bcl-x_L Protein Is Associated with VCD-Induced Ovotoxicity in Rats. Biology of Reproduction. 65(5): 1489-1495.

Hu, X, Christian, PJ, Thompson, KE, Sipes, IG, and Hoyer, PB. 2001b. Apoptosis induced in rats by 4-vinylcyclohexene diepoxide is associated with activation of the caspase cascades. *Biology of Reproduction*. 65: 87-93.

Hughes, FM and Gorospe, WC. 1991. Biochemical identification of apoptosis (programmed cell death) in granulosa cells: evidence for a potential mechanism underlying follicular atresia. *Endocrinology*. 129(5): 2415-2422.

IARC (International Agency for Research on Cancer). 1994a. 4-Vinylcyclohexene. In: *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: Some Industrial Chemicals*, vol. 60, Lyon, France; 347-359.

IARC (International Agency for Research on Cancer). 1994b. 4-Vinylcyclohexene diepoxide. In: *IARC Monographs of the Evaluation of Carcinogenic Risks to Humans: Some Industrial Chemicals*, vol. 60, Lyon, France; 361-372.

Inkster, SE and Brodie, AM. 1991. Expression of aromatase cytochrome P-450 in premenopausal and postmenopausal human ovaries: an immunocytochemical study. *Journal of Clinical Endocrinology and Metabolism*. 73: 717-726.

Iwai, T, Nanbu, Y, Iwai, M, Taii, S, Fujii, S, Mori, T. 1990. Immunohistochemical localization of oestrogen receptors and progesterone receptors in the human ovary throughout the menstrual cycle. *Virchows Archiv - A, Pathology Anatomy & Histopathology*. 417: 369-375.

Jarrell, JF, McMahon, A, Villeneuve, D, Franklin, C, Singh, A, Vallie, VE, Bartlett, S. 1993. Hexachlorobenzene toxicity in the monkey primordial germ cell with induced porphyria. *Reproductive Toxicology*. 7(1): 41-47.

Jenster, G. 1998. Coactivators and corepressors as mediators of nuclear receptor function: an update. *Molecular and Cellular Endocrinology*. 143(1-2): 1-7.

Jeong, HG and Lee, SS. 1998. Suppressive effects of estradiol on 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated transcriptional activation of murine *Cyp1a-1* in mouse hepatoma Hepa 1c1c7 cells. *Cancer Letters*. 133: 177-184.

Jeong, JH, Kwon, ES, and Roe, JH. 2001. Characterization of the manganese-containing superoxide dismutase and its gene regulation in stress response of schizosaccharomyces pombe. *Biochemical and Biophysical Research Communications*. 283: 908-914.

Joakimsen, O, Bonaa, KH, Stensland-Bugge, E, and Jacobsen, BK. 2000. Population-based study of age at menopause and ultrasound assessed carotid atherosclerosis: The Thromso Study. *Journal of Clinical Epidemiology*. 53(5): 525-530.

Johannisson, R, and Ocker, H. 1997. Cyclophosphamide-induced aberrations of chromosome pairing in pachytene oocytes. *Mutation Research*. 374(2): 185-192.

Kaipia, A and Hsueh, AJW. 1997. Regulation of ovarian follicle atresia. *Annual Reviews of Physiology*. 59: 349-363.

Kao, S-W, Sipes, IG, and Hoyer, PB. (1999). Early effects of ovotoxicity induced by 4-vinylcyclohexene diepoxide in rats and mice. *Reproductive Toxicology*. 13: 65-75.

Katzenellenbogen, BS. 1996. Estrogen receptors: bioactivities and interactions with cell signaling pathways. *Biology of Reproduction*. 54: 287-293.

Kharat, I and Saatcioglu, F. 1996. Antiestrogenic effects of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin are mediated by direct transcriptional interference with the liganded estrogen receptor. *Journal of Biological Chemistry*. 271(18): 10533-10537.

Klinge, CM, Bowers, JL, Kulakosky, PC, Kamboj, KK, and Swanson, HI. 1999. The aryl hydrocarbon receptor (AHR)/AHR nuclear translocator (ARNT) heterodimer interacts with naturally occurring estrogen response elements. *Molecular and Cellular Endocrinology*. 157: 105-119.

Klinge, CM, Kaur, K, and Swanson, HI. 2000. The aryl hydrocarbon receptor interacts with estrogen receptor alpha and orphan receptors COUP-TF1 and ARRa1. *Archives of Biochemistry and Biophysics*. 373(1): 163-174.

Korach, KS. 2000. Estrogen receptor knock-out mice: molecular and endocrine phenotypes. *Journal for the Society for Gynecologic Investigation*. 7(1, supplement): S16-17.

Krege, JH, Hodgin, JB, Couse, JF, Enmark, E, Warner, M, Mahler, JF, Sar, M, Korach, KS, Gustafsson, J-A, and Smithies, O. 1998. Generation and reproductive phenotypes of mice lacking estrogen receptor β. *Proceedings of the National Academy of Sciences of the USA*. 95: 15677-15682.

Krishnan, V, Porter, W, Santostefano, M, Wang, X, and Safe, S. 1995. Molecular mechanism of inhibition of estrogen-induced cathepsin D gene expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in MCF-7 cells. *Molecular and Cellular Biology*. 15(12): 6710-6719.

Kroemer, G., Dallaporta, B., and Resche-Rigon, M. 1998. The mitochondrial death/life regulator in apoptosis and necrosis. *Annual Review of Physiology*. 60: 619-642.

Krust, A., Green, S., Argos, P., Kumar, V., Walter, P., Bornert, J.M., and Chambon, P. 1986. The chicken oestrogen receptor sequence: homology with v-erbA and the human oestrogen and glucocorticoid receptors. *EMBO Journal*. 5: 891-897.

Kuiper, G.G.J.M., Enmark, E., Pelto-Huikko, M., Nilsson, S., and Gustafsson, JA. 1996. Cloning of a novel receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences of the USA*. 93(12): 5925-5930.

Kuiper, G.G.J.M., Carlsson, B., Grandien, K., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J-A. 1997. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology*. 138(3): 863-870.

Kuiper, G.G.J.M. and Gustafsson, J-A. 1997. The novel estrogen receptor-β subtype: potential role in the cell- and promoter-specific actions of estrogens and anti-estrogens. *Federation of European Biochemical Societies*. 410: 87-90.

Kuiper, G.G.J.M., Lemmen, J.G., Carlsson, B., Corton, J.C., Safe, S.H., Van Der Saag, P.T., Van Der Burg, B., and Gustafsson, J-A. 1998. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor b. *Endocrinology*. 139(10): 4252-4263.

Lahvis, GP and Bradfield, CA. 1998. *Ahr* Null Alleles: Distinctive or Different? *Biochemical Pharmacology*. 56: 781-787.

Lane, JM and Nydick, M. 1999. Osteoporosis: current models of prevention and treatment. *Journal of the American Academy of Orthopaedic Surgeons*. 7(1): 19-31.

LaPres, JJ, Glover, E, Dunham, EE, Bunger, MK, and Bradfield, CA. 2000. ARA9 modifies agonist signaling through an increase in cytosolic aryl hydrocarbon receptor. *Journal of Biological Chemistry*. 275(9): 6153-6159.

Lee, MW, Park, SC, Chae, HS, Bach, JH, Lee, HJ, Lee, SH, Kang, YK, Kim, KY, Lee, WB, and Kim, SS. 2001. The protective role of HSP90 against 3-hydroxykynurenine-induced neuronal apoptosis. *Biochemical and Biophysical Research Communincations*. 284(2): 261-267.

Li, P, Nijhawan, D, Budihardjo, I, Srinivasula, SM, Ahmad, M, Alnemri, ES, and Wang, X. 1997. Cytochrome *c* and dATP-dependent formation of Apaf-1/caspase-9 complex initiates an apoptotic protease cascade. *Cell*. 94: 491-501.

Lindros, KO, Oinonen, T, Kettunen, E, Sippel, H, Muro-Lupori, C, and Koivusalo, M. 1998. Aryl hydrocarbon receptor-associated genes in rat liver. *Biochemical Pharmacology*. 55: 413-421.

Lubahn, DB, Moyer, JS, Golding, TS, Couse, JF, Korach, KS, and Smithies, O. 1993. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences of the United States of America*. 90: 11 162-166.

Lunenfeld, B and Eshkol, A. 1969. Immunology of follicle stimulating hormone and luteinizing hormone. *Vitamins and Hormones*. 27: 131-197.

Lunenfeld, B and Insler, V. 1993. Follicular development and its control. *Gynecological Endocrinology*. 7: 285-291.

Lutz, CT, Browne, G, and Petzold, CR. 1998. Methylcholanthrene causes increased thymocyte apoptosis. *Toxicology*. 128(2): 151-167.

Ma, Q and Whitlock, JP Jr. 1996. The aromatic hydrocarbon receptor modulates the Hepa 1c1c7 cell cycle and differentiated state independently of dioxin. *Molecular and Cellular Biology*. 16(5): 2144-2150.

Maenpaa, J, Hall, SD, Ring, BJ, Strom, SC, and Wrighton, SA. 1998. Human cytochrome P450 3A (CYP3A) mediated midazolam metabolism: the effect of assay conditions and regioselective stimulation by alpha-naphthoflavone, terfenadine and testosterone. *Pharmacogenetics*. 8: 137-155.

Martucci, CP and Fishman, J. 1993. P450 enzymes of estrogen metabolism. *Pharmacology & Therapeutics*. 57(2-3): 237-257.

Masten, Y and Gary, A. 1999. Is anyone listening? Does anyone care? Menopausal and postmenopausal health risks, outcomes, and care. *Nurse Practitioner Forum*. 10(4): 195-200.

Matikainen, T, Perez, GI, Jurisicova, A, Pru, JK, Schlezinger, JJ, Ryu, HY, Laine, J, Sakai, T, Korsmeyer, SJ, Casper, RF, Sherr, DH, and Tilly, JL. 2001. Aromatic hydrocarbon receptor-driven Bax gene expression is required for premature ovarian failure caused by biohazardous environmental chemicals. *Nature Genetics*. 28(4): 355-360.

Mattison, DR, and Thorgeirsson, SS. 1978. Gonadal aryl hydrocarbon hydroxylase in rats and mice. *Cancer Research*. 38: 1368-1373.

Mattison, DR and Thorgeirsson, SS. 1979. Ovarian aryl hydrocarbon hydroxylase activity and primordial oocyte toxicity of polycyclic aromatic hydrocarbons in mice. *Cancer Research*. 39: 3471-3475.

Mattison, D.R. and Schulman, J.D. 1980. How xenobiotic compounds can destroy oocytes. *Conomporary Obstetrics and Gynecology*. 15: 157-169.

Mattison, DR. 1980. Morphology of oocyte and follicle destruction by polycyclic aromatic hydrocarbons in mice. *Toxicology & Applied Pharmacology*. 53(2): 249-59.

Mattison DR. 1982. The effects of smoking on fertility from gametogenesis to implantation. *Environmental Research*. 28(2): 410-33.

Mattison, DR, Plowchalk, BS, Meadows, MJ, Miller, MM, Malek, A, and London, S. 1989. The effect of smoking on oogenesis, fertilization, and implantation. *Seminars in Reproductive Endocrinology*. 7(4): 291-304.

Mayer, LP, Kroeger, N, Christian, PJ, Marion, SL, and Hoyer, PB. 2001. Long-term effects of ovotoxicity in rats exposed to 4-vinylcyclohexene diepoxide. *Biology of Reproduction*. 64(Supp 1): 192.

McGee, EA, Hsu, S-Y, Kaipia, A, and Hseuh, AJW. 1998. Cell death and survival during ovarian follicle development. *Molecular and Cellular Endocrinology*. 140: 15-18.

Messina, MJ, Persky, V, Setchell, KDR, and Barnes, S. 1994. Soy intake and cancer risk: a review of the *in vitro* and *in vivo* data. *Nutrition and Cancer*. 21: 113-131.

Monniaux, D, Huet, C, Besnard, N, Clement, F, Bosc, M, Pisselet, C, Monget, P, and Mariana, JC. 1997. Follicular growth and ovarian dynamics in mammals. *Journal of Reproduction and Fertility*. Supplement S1: 3-23.

Montano, M.M., Jaiswal, A.K., and Katzenellenbogen, B.S. 1998. Transcriptional regulation of the human quinone reductase gene by antiestrogen-liganded estrogen receptor-alpha and estrogen receptor-beta. *Journal of Biological Chemistry*. 273(39): 25443-25449.

Moran, FM, Enan, E, Vandervoort, CA, Stewart, DR, Conley, AJ, Overstreet, JW, and Lasley, BL. 1997. 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) effects on steroidogenesis of human luteinized granulosa cells *in vitro*. Society for the Study of Reproduction, Portland, OR, 1997, [Suppl 1] 56 (Abstract 65).

Morrow NL. 1990. The industrial production and use of 1,3-butadiene. *Environmental Health Perspectives*. 86: 7-8.

Motyl, T. 1999. Regulation of apoptosis: involvement of Bcl-2-related proteins. *Reproduction, Nutrition, Development*. 39(1): 49-59.

Murdoch, WJ. 1998. Inhibition by oestradiol of oxidative stress-induced apoptosis in pig ovarian tissues. *Journal of Reproduction and Fertility*. 114: 127-130.

Nathan, L and Chaudhuri, G. 1998. Antioxidant and prooxidant actions of estrogens: potential physiological and clinical implications. *Seminars in Reproductive Endocrinology*. 16: 309-314.

National Toxicology Program (NTP). 1982. *Bioassay of 2,3,7,8-tetrachlorodibenzo-p-dioxin for possible carcinogenicity (gavage study)*. NTP technical report No. 209. U.S. Department of Health and Human Services. Public Health Service. National Institutes of Health. Public Information. National Toxicology Program, Research Triangle, N.C.

National Toxicology Program (NTP). 1986. *Toxicology and carcinogenesis studies of 4-vinyl-1-cyclohexene in F344/N rats and B6C3F$_1$ mice*. NTP technical report No. 303. U.S. Department of Health and Human Services. Public Health Service. National Institutes of Health. Public Information. National Toxicology Program, Research Triangle, N.C.

National Toxicology Program (NTP). 1989. *Toxicology and carcinogenesis studies of 4-vinyl-1-cyclohexene diepoxide in F344/N rats and B6C3F$_1$ mice.* NTP technical report No. 303. U.S. Department of Health and Human Services. Public Health Service. National Institutes of Health. Public Information. National Toxicology Program, Research Triangle, N.C.

National Toxicology Program (NTP). 1993. *Toxicology and carcinogenesis studies of 1,3-butadiene in B6C3F$_1$ mice.* NTP technical report No. 434. U.S. Department of Health and Human Services. Public Health Service. National Institutes of Health. Public Information. National Toxicology Program, Research Triangle, N.C.

Near, RI, Matulka, RA, Mann, KK, Gogate, SU, Trombino, AF, and Sherr, DH. 1999. Regulation of preB cell apoptosis by aryl hydrocarbon receptor/transcription factor-expressing stromal/adherent cells. *Proceedings of the Society for Experimental Biology and Medicine.* 221(3): 242-252.

Nebert, DW and Gelboin, HV. 1969. The in vivo and in vitro induction of the aryl hydrocarbon hydroxylase in mammalian cells of different species, tissues, strains, and developmental and hormonal states. *Archives of Biochemistry and Biophysics.* 134: 76-89.

Nebert, DW, Roe, AL, Dieter, MZ, Solis, WA, Yang, Y, and Dalton, TP. 2000. Role of the aromatic hydrocarbon receptor and [*Ah*] gene battery in the oxidative stress response, cell cycle control, and apoptosis. *Biochemical Pharmacology.* 59: 65-85.

Nelson, JF, Gosden, RG, and Felicio, LS. 1985. Effect of dietary restriction on estrous cyclicity and follicular reserves in ageing C57BL/6J mice. *Biology of Reproduction.* 32: 515-522.

Nguyen, TA, Hoivik, D, Lee, J-E, Safe, S. 1999. Interactions of nuclear receptor coactivator/corepressor proteins with the aryl hydrocarbon receptor complex. *Archives of Biochemistry and Biophysics.* 367(2): 250-257.

Ohsako, S, Bunick, D, and Hayashi, Y. 1995. Immunocytochemical observation in the 90 kD heat shock protein (HSP90): high expression in primordial and pre-meiotic germ cells of male and female rat gonads. *The Journal of Histochemistry and Cytochemistry.* 43: 67-76.

Okayasu, I, Kong, YM, and Rose, NR. 1981. Effect of castration and sex hormones on experimental autoimmune thyroiditis. *Clinical Immunology and Immunopathology.* 20: 240-245.

Paganini-Hill, A and Henderson, VW. 1996. Estrogen replacement therapy and risk of Alzheimer disease. *Archives of Internal Medicine*. 156(19): 2213-2217.

Pandey, P, Saleh, A, Nakazawa, A, Kumar, S, Srinivasula, SM, Kumar, V, Weichselbaum, R, Nalin, C, Alnemri, ES, Kufe, D, and Kharbanda, S. 2000. Negative regulation of cytochrome c-mediated oligomerization of Apaf-1 and activation of Procaspase-9 by heat shock protein 90. *EMBO Journal*. 19(16): 4310-4322.

Parini, P, Angelin, B, Stavreus-Evers, A, Freyschuss, B, Eriksson, H, and Rudling, M. 2000. Biphasic effects of the natural estrogen 17β-estradiol on hepatic cholesterol metabolism in intact female rats. *Arteriosclerosis, Thrombosis, and Vascular Biology*. 20: 1817-1823.

Parker, MG. 1995. Structure and function of estrogen receptors. *Vitamins and Hormones*. 51: 267-287.

Parker, M.G. 1998. Transcriptional activation by oestrogen receptors. *Biochemical Society Symposia*. 63: 45-50.

Patrone, C, Andersson, S, Korhonen, L, and Lindholm, D. 1999. Estrogen receptor-dependent regulation of sensory neuron survival in developing dorsal root ganglion. *Proceedings of the National Academy of Sciences of the United States of America*. 96: 10905-10910.

Paulson, KE. 1989. Xenobiotic regulation of glutathione *S*-transferase Ya gene expression. *Molecular Toxicology*. 2: 215-235.

Pederson, T and Peters, H. 1968. Proposal for the classification of oocytes and follicles in the mouse ovary. *Journal of Reproduction and Fertility*. 17: 555-557.

Pelzer, T, Schumann, M, Neumann, M, deJager, T, Stimpel, M, Serfling, E, and Neyses, L. 2000. 17β-estradiol prevents programmed cell death in cardiac myocytes. *Biochemical and Biophysical Research Communications*. 268: 192-200.

Perez, G.I., Knudson, C.M., Leykin, L., Korsmeyer, S.J., and Tilly, J.L. 1997. Apoptosis-associated signaling pathways are required for chemotherapy-mediated female germ cell destruction. *Nature Medicine*. 3: 1228-32.

Petersen, DN, Tkalcevic, GT, Koza-Taylor, PH, Turi, TG, and Brown, TA. 1998. Identification of estrogen receptor beta2, a functional variant of estrogen receptor beta expressed in normal rat tissues. *Endocrinology*. 139(3): 1082-1092.

Pettersson, K, Grandien, K, Kuiper, GG, and Gustafsson, JA. 1997. Mouse estrogen receptor beta forms estrogen response element-binding heterodimers with estrogen receptor alpha. *Molecular Endocrinology*. 11(10): 1486-1496.

Picton, H, Briggs, D, and Gosden, R. 1998. The molecular basis of oocyte growth and development. *Molecular and Cellular Endocrinology*. 145: 27-37.

Piechocki, MP and Hines, RN. 1998. Functional characterization of the human *CYP1A1* negative regulatory element: Modulation of Ah receptor-mediated transcriptional activity. *Carcinogenesis*. 19: 771-780.

Pike, CJ. 1999. Estrogen modulates neuronal Bcl-X_L expression and beta-amyloid-induced apoptosis: relevance to Alzheimer's disease. *Journal of Neurochemistry*. 72: 1552-1563.

Pitt, JA, Feng, L, Abbott, BD, Schmid, J, Batt, RE, Costich, TG, Koury, ST, and Bofinger, DP. 2001. Expression of AhR and ARNT mRNA in cultured human endometrial explants exposed to TCDD. *Toxicological Sciences*. 62: 289-298.

Plowchalk, DR and Mattison, DR. 1992. Reproductive toxicity of cyclophosphamide in the c57BL/6N mouse: 1. Effects on ovarian structure and function. *Reproductive Toxicology*. 6: 411-421.

Poland, A, Glover, E, Robinson, JR, and Nebert, DW. 1974. Genetic expression of aryl hydrocarbon hydroxylase activity: induction of monooxygenase activities and cytochrome P1-450 formation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice genetically "nonresponsive" to other aromatic hydrocarbons. *Journal of Biological Chemistry*. 249: 5599-5606.

Polkowski, K and Mazurek, AP. 2000. Biological properties of genistein. A review of in vitro and in vivo data. *Acta Poloniae Pharmaceutica*. 57: 135-155.

Ratkay, LG, Zhang, D, Tonzetich, J, Levy, JG, and Waterfield, JD. 1994. Evaluation of a model of post-partum arthritis and the role of oestrogen in prevention of MRL-lpr/lpr associated rheumatic conditions. *Clinical and Experimental Immunology*. 98: 52-59.

Reiners, JJ and Clift, RE. 1999. Aryl hydrocarbon receptor regulation of ceramide-induced apoptosis in murine hepatoma 1c1c7 cells. A function independent of aryl hydrocarbon receptor nuclear translocator. *Journal of Biological Chemistry*. 274(4): 2502-2510.

Ricci, MS, Toscano, DG, Mattingly, CJ, Toscano, WA. 1999. Estrogen receptor reduces cyp1A1 induction in cultured human endometrial cells. *Journal of Biological Chemistry*. 274(6): 3430-3438.

Richards, JS. 1980. Maturation of ovarian follicles; actions and interactions of pituitary and ovarian hormones on follicular cell differentiation. *Physiological Reviews*. 60: 51-89.

Robles, R, Morita, Y, Mann, KK, Perez, GI, Yang, S, Matikainen, T, Sherr, DH, and Tilly, JL. 2000. The aryl hydrocarbon receptor, a basic helix-loop-helix transcription factor of the PAS gene family, is required for normal ovarian germ cell dynamics in the mouse. *Endocrinology*. 141(1): 450-453.

Roe, SM, Prodromou, C, O'Brien, R, Ladbury, JE, Piper PW, and Pearl, LH. 1999. Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *Journal of Medicinal Chemistry*. 42: 260-266.

Rohrdanz, E, Schmuck, G, Ohler, S, and Kahl, R. 2001. The influence of oxidative stress on catalase and MnSOD gene transcription in astrocytes. *Brain Research*. 900: 128-136.

Safe, S. 1990. Polychlorinated biphenyls (PCBs), dibenzo-p-dioxins (PCDDs), dibenzofurans (PCDFs), and related compounds: environmental and mechanistic considerations which support the developmental of toxic equivalency factors (TEFs). *Critical Reviews of Toxicology*. 21: 51-88.

Safe, S, Astroff, B, Harris, M, Zacharewski, T, Dickerson, R, Romkes, M, and Biegel, L. 1991. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) and related compounds as antioestrogens: characterization and mechanism of action. *Pharmacology and Toxicology*. 69(6): 400-9.

Safe, S. 1995. Modulation of gene expression and endocrine response pathways by 2,3,7,8-tetrachlorodibenzo-p-dioxin and related compounds. *Pharmacology and Therapeutics*. 67: 247-81.

Safe, S, Wang, F, Porter, R, Duan, R, and McDougal, A. 1998. Ah receptor agonists as endocrine disruptors: antiestrogenic activity and mechanisms. *Toxicology Letters*. 102-103: 343-347.

Schneider, C, Sepp-Lorenzino, L, Nimmesgern, E, Ouerfelli, O, Danishefsky, S, Rosen, N, and Hartl, FU. 1996. Pharmacologic shifting of a balance between protein refolding and degradation mediated by Hsp90. *Proceedings of the National Academy of Sciences of the United States of America*. 93: 14536-14541.

Schmidt, JV and Bradfield, CA. 1996. Ah receptor signaling pathways. *Annual Review of Cell and Developmental Biology*. 12: 55-89.

Schmidt, JV, Su, GH, Reddy, JK, Simon, MC, and Bradfield, CA. 1996. Characterization of a murine *Ahr* null allele: Involvement of the Ah receptor in hepatic growth and development. *Proceedings of the National Academy of Sciences of the United States of America.* 93: 6731-6736.

Schor, NF, Tyurina, YY, Tyurin, VA, and Kagan, VE. 1999. Differential membrane antioxidant effects of immediate and long-term estradiol treatment of MCF-7 breast cancer cells. *Biochemical and Biophysical Research Communications.* 260: 410-5.

Schrenk, D. 1998. Impact of dioxin-type induction of drug-metabolizing enzymes on the metabolism of endo- and xenobiotics. *Biochemical Pharmacology.* 55: 1155-62.

Segntiz, B and Gehring, U. 1997. The function of steroid hormone receptors is inhibited by the hsp90-specific compound geldanamycin. *Journal of Biological Chemistry.* 272: 18694-18701.

Shiromizu, K, Thorgeirsson, SS, and Mattison, DR. 1984. Effect of cyclophosphamide on oocyte and follicle number in sprague-dawley rats, C57BL/6N and DBA/2N mice. *Pediatric Pharmacology.* 4(4): 213-21.

Silbergeld, EK and Mattison, DR. 1987. Experimental and clinical studies on the reproductive toxicology of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *American Journal of Industrial Medicine.* 11: 131-44.

Singer, CA, Rogers, KL, and Dorsa, DM. 1998. Modulation of Bcl-2 expression: a potential component of estrogen protein in NT2 neurons. *NeuroReport.* 9: 2565-2568.

Smith, BJ, Sipes, IG, Stevens, JC, and Halpert, JR. 1990a. The biochemical basis for the species difference in hepatic microsomal 4-vinylcyclohexene epoxidation between female mice and rats. *Carcinogenesis.* 11: 1951-57.

Smith, BJ, Mattison, DR, and Sipes, IG. 1990b. The role of epoxidation in 4-vinylcyclohexene-induced ovarian toxicity. *Toxicology and Applied Pharmacology.* 105: 372-81.

Smith, BJ, Carter, DE, and Sipes, IG. 1990c. Comparison of the disposition and *in vitro* metabolism of 4-vinylcyclohexene in the female mouse and rat. *Toxicology and Applied Pharmacology.* 105: 364-71.

Smith, EP, Boyd, J, Frank, GR, Takahashi, H, Cohen, RM, Specker, B, Williams, TC, Lubahn, DB, and Korach, KS. 1994d. Estrogen resistance caused by a mutation in the estrogen receptor gene in a man. *New England Journal of Medicine.* 331: 1088-89.

Son, DS, Ushinohama, K, Gao, X, Taylor, CC, Roby, KF, Rozman, KK, and Terranova, PF. 1999. 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) blocks ovulation by a direct action on the ovary without alteration of ovarian steriodogenesis: lack of a direct effect on ovarian granulosa and thecal-interstitial cell steriodogenesis *in vitro*. *Reproductive Toxicology*. 13(6): 521-530.

Sowers, MFR and LaPietra, MT. 1995. Menopause: It's epidemiology and potential association with chronic diseases. *Epidemiological Reviews*. 17(2): 287-302.

Springer, LN, Flaws, JA, Sipes, IG, and Hoyer, PB. 1996a. Follicular mechanisms associated with 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. *Reproductive Toxicology*. 10(2): 137-43.

Springer, L.N., Tilly, J.L., Sipes, I.G., and Hoyer, P.B. 1996b. Enhanced expression of *bax* in small preantral follicles during 4-vinylcyclohexene diepoxide-induced ovotoxicity in the rat. *Toxicology and Applied Pharmacology*. 139: 402-410.

Springer, LN, McAsey, ME, Flaws, JA, Tilly, JL, Sipes, IG, and Hoyer, PB. 1996c. Involvement of apoptosis in 4-vinylcyclohexene diepoxide-induced ovotoxicity in rats. *Toxicology and Applied Pharmacology*. 139: 394-401.

Spyridopoulos, I., Sullivan, A.B., Kearney, M., Isner, J.M., and Losordo, D.W. 1997. Estrogen-receptor-mediated inhibition of human endothelial cell apoptosis. Estradiol as a survival factor. *Circulation*. 95(6): 1505-14.

Strigard, K, Holmdahl, R, and Olsson, T. 1990. Oestrogen treatment reduces disease duration of experimental allergic neuritis in rats and suppresses T cell response to myelin. *Acta Neurologica Scandinavica*. 81: 436-442.

Sugita, T, Tanaka, S, Murakami, T, Miyushi, H, and Obnuki, T. 1999. Immunosuppressive effects of the heat shock protein 90-binding antibiotic geldanamycin. *Biochemistry and Molecular Biology International*. 47(4): 587-595.

Tam, PP and Liu, WK. Gonadal development and fertility of mice treated prenatally with cadmium during the early organogenesis stages. *Teratology*. 32(3): 453-462.

Teixeira, C, Reed, JC, and Pratt, MAC. 1995. Estrogen promotes hemotherapeutic drug resistance by a mechanism involving bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Research*. 55: 3902-3907.

Telleria, CM, Zhong, L, Deb, S, Srivastava, RK, Park, KS, Sugino, N, Park-Sarge, O-K, and Gibori, G. 1998. Differential expression of the estrogen receptors alpha and beta in the rat corpus luteum of pregnancy: regulation by prolactin and placental lactogens. *Endocrinology*. 139: 2432-2442.

Thomsen, JS, Wang, X, Hines, RN, and Safe, S. 1994. Restoration of aryl hydrocarbon (Ah) responsiveness in MDA-MD-231 human breast cancer cells by transient expression of the estrogen receptor. *Carcinogenesis*. 15: 933-937.

Tilly, JL, Billig, H, Kowalski, KI, and Hsueh, AJW. 1992. Epidermal growth factor and basic fibroblast growth factor suppress the spontaneous onset of apoptosis in cultured rat granulosa cells and follicles by a tyrosine kinase-dependent mechanism. *Molecular Endocrinology*. 6: 1942-1950.

Tilly, JL. 1998. Molecular and genetic basis of normal and toxicant-induced apoptosis in female germ cells. Toxicology Letters. 102-103: 497-501.

Tomkinson, A., Reeve, J., Shaw, R.W., and Noble, B.S. 1997. The death of osteocytes via apoptosis accompanies estrogen withdraw in human bone. *Journal of Clinical Endocrinology and Metabolism*. 92(9): 3128-3135.

Tsujimoto, Y and Shimizu, S. 2000. Bcl-2 family: life-or-death switch. *FEBS Letters*. 466(1): 6-10.

Tuohimaa, P, Pekki, A, Blauer, M, Joensuu, T, Vilja, P, and Ylikomi, T. 1993. Nuclear progesterone receptor is mainly heat shock protein 90-free in vivo. *Proceedings of the National Academy of Sciences of the United States of America*. 90: 5848-5852.

Uilenbroek, JT, Meijs-Roelofs, HM, de Greef, WJ, and de Jong, FH. 1975. Proceedings: Gonadotrophin, oestradiol-17beta and progesterone levels around the time of first ovulation in the rat. *Journal of Endocrinology*. 64: 42P-43P.

Valanis, B, Vollmer, W, Labuhn, K, and Glass, A. 1997. Occupational exposure to antineoplastic agents and self-reported infertility among nurses and pharmacists. *Journal of Occupational & Environmental Medicine*. 39(6): 574-80.

Vegeto, E, Pollio, G, Pellicciari, C, and Maggi, A. 1999. Estrogen and progesterone induction of survival of monoblastoid cells undergoing TNF-α-induced apoptosis. *FASEB Journal*. 13: 793-803.

Vessey, M, Baron, J, Doll, R, McPherson, K, and Yeates, D. 1983. Oral contraceptives and breast cancer: final report of an epidemiological study. *British Journal of Cancer*. 47(4): 455-462.

Vickers, PJ, Dufresne, MJ, and Cowan, KH. 1989. Relation between cytochrome P450 1A1 expression and estrogen receptor content of human breast cancer cells. *Molecular Endocrinology*. 3: 157-64.

Waksman, Y, Hod, I, and Friedman, A. 1996. Therapeutic effects of estradiol benzoate on development of collagen-induced arthritis (CIA) in the Lewis rat are mediated via suppression of the humoral response against denatured collagen type II (CII). *Clinical and Experimental Immunology*. 103: 376-383.

Wang, TT and Phang, JM. 1995. Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Research*. 55(12): 2487-89.

White, TEK and Gasiewicz, TA. 1993. The human estrogen receptor structural gene contains a DNA sequence that binds activated mouse and human Ah receptors: a possible mechanism of estrogen receptor regulation by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. *Biochemical and Biophysical Research Communications*. 193(3): 956-62.

Wyllie, AH, Kerr, JF, and Currie, AR. 1980. Cell death: the significance of apoptosis. *International Review of Cytology*. 68: 251-306.

Wyllie, AH. 1987. Apoptosis: cell death in tissue regulation. *Journal of Pathology*. 153(4): 313-6.

Xie, YQ, Takimoto, K, Pitot, HC, Miskimins, WK, and Lindahl, R. 1996. Characterization of the rat Class 3 aldehyde dehydrogenase gene promoter. *Nucleic Acids Research*. 24: 4185-91.

Ylikomi, T, Wurtz, J-M, Syvala, H, Passinen, S, Pekki, A, Haverinen, M, Blauer, M, Tuohimaa, P, and Gronemeyer, H. 1998. Reappraisal of the role of heat shock proteins as regulators of steroid receptor activity. *Critical Reviews in Biochemistry and Molecular Biology*. 33(6): 437-66.

Yu, X, Kamijima, M, Ichihara, G, Li, W, Kitoh, J, Xie, Z, Shibata, E, Hisanaga, N, and Takeuchi, Y. 1999. 2-Bromopropane causes ovarian dysfunction by damaging primordial follicles and their oocytes in female rats. *Toxicology and Applied Pharmacology*. 159(3): 185-93.

Yu, CL, Driggers, P, Barrera-Hernandez, G, Nunez, SB, Segars, JH, and Cheng, S. 1997. The tumor suppressor p53 is a negative regulator of estrogen receptor signaling pathways. *Biochemical and Biophysical Research Communications*. 239(2): 617-620.

Zacharewski, T, Harris, M, and Safe, S. 1991. Evidence for the mechanism of action of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin-mediated decrease of nuclear estrogen receptor levels in wild-type and mutant mouse Hepa 1c1c7 cells. *Biochemical Pharmacology*. 41(12): 1931-9.

Zaher, H., Fernandez-Salguero, PM, Letterio, J, Sherikh, MS, Fornace, AJ, Roberts, AB, and Gonzalez, FJ. 1998. The involvement of the aryl hydrocarbon receptor in the activation of transforming growth factor-beta and apoptosis. *Molecular Pharmacology.* 54(2): 313-21.