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Bax, Caspase-2, and Caspase-3 Are Required for Ovarian Follicle Loss Caused by 4-Vinylcyclohexene Diepoxide Exposure of Female Mice *in Vivo*

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The industrial chemical, 4-vinylcyclohexene diepoxide (VCD), kills oocytes within immature follicles in the ovaries of mice and rats and is considered a potential occupational health hazard. It has been reported that VCD-induced follicle loss occurs via a cell death process involving elevated expression of Bax, a proapoptotic Bcl-2 family member, and increased caspase-3-like activity. We have previously shown that oocytes lacking acid sphingomyelinase (ASMase; an enzyme that generates the proapoptotic stress sensor ceramide), the aromatic hydrocarbon receptor (Ahr), Bax, or caspase-2 are resistant to apoptosis induced by other chemical toxicants. Therefore, this study was designed to investigate the functional importance of ASMase, Ahr, Bax, and caspase-2 as well as the related executioner enzyme caspase-3 to VCD-induced ovotoxicity in mice using gene knockout technology. For each gene mutant mouse line, wild-type and homozygous-null female siblings derived from heterozygous matings were given once-daily ip injections of either vehicle (sesame oil) or VCD (80 mg/kg body weight) for 15 d (three or four mice per treatment group per genotype). Ovaries were collected 24 h after the final injection and analyzed for the total number of nonatretic primordial and primary follicles remaining per ovary. No differences in the extent of primordial or primary follicle destruction resulting from VCD exposure were observed in wild-type *vs.* ASMase- or Ahr-deficient mice. By contrast, the extent of VCD-induced primordial follicle depletion in Bax-deficient mice (45 ± 11%) was significantly ($P < 0.05$) lower than that in wild-type females (85 ± 2%). The extent of primary follicle loss in *bax*-null mice exposed to VCD (3 ± 22%) was also significantly ($P < 0.05$) lower than that in their wild-type sisters (86 ± 4%). In caspase-2-deficient mice, significantly ($P < 0.05$) fewer oocyte-containing primary follicles were destroyed by VCD (17 ± 19%) *vs.* wild-type controls (71 ± 6%); however, no significant difference in the extent of VCD-induced primordial follicle destruction was observed in *caspase-2*-null *vs.* wild-type females. Finally, in caspase-3-deficient mice, significantly ($P < 0.05$) fewer oocyte-containing primary follicles were destroyed by VCD (33 ± 3%) *vs.* wild-type controls (71 ± 2%); however, no significant difference in the extent of VCD-induced primordial follicle destruction was observed in *caspase-3*-null *vs.* wild-type females. We conclude that Bax, caspase-2, and caspase-3, but not ASMase or Ahr, are functionally important in VCD-induced follicle loss. However, as a loss of Bax, caspase-2, or caspase-3 function conveyed only partial protection from the ovotoxic effects of VCD, other cell death pathways that either function independently of Bax, caspase-2, and caspase-3 or are not apoptotic in nature are also involved. (*Endocrinology* 144: 69–74, 2003)

STUDIES OF GENE mutant mice lacking various regulators or executioners of the programmed cell death pathway of apoptosis have provided important insight into the mechanisms responsible for normal and pathological oocyte depletion underlying ovarian failure (1, 2). For example, mice harboring a targeted disruption in the gene encoding Bax, a key proapoptotic member of the Bcl-2 family of cell death modulators (3, 4), exhibit a reduced rate of primordial and primary follicle atresia, leading to a striking prolongation of the ovarian life span into advanced chronological age (5). Oocytes of Bax-deficient female mice are also resistant to developmental death during gametogenesis (6) and to apoptosis induced by both clinical (*e.g.* doxorubicin) (7) and environmental [*e.g.* polycyclic aromatic hydrocarbons (PAH)] (8, 9) toxicants. However, Bax deficiency does not rescue oocytes from death caused by meiotic recombination defects associated with *ataxia telangiectasia-mutated* (*Atm*) gene inactivation (10), suggesting the existence of more than one cell death pathway that functions to delete oocytes exposed to various lethal stimuli.

Upstream of Bax in some programmed cell death signaling pathways, a role for the proapoptotic messenger ceramide (11) has been proposed from studies of mutant mice lacking the ceramide-generating enzyme, acid sphingomyelinase (ASMase). These mice are born with a significantly larger reserve of primordial oocytes due to a germ cell autonomous death defect that leads to germline hyperplasia during gametogenesis (12). Moreover, ASMase-deficient oocytes are

Abbreviations: Ahr, Aromatic hydrocarbon receptor; ASMase, acid sphingomyelinase; *Atm*, *ataxia telangiectasia-mutated* (gene); PAH, polycyclic aromatic hydrocarbon; VCD, 4-vinylcyclohexene diepoxide.

completely resistant to apoptosis induced by the widely used chemotherapeutic drug, doxorubicin (12). These findings together with observations that ceramide and Bax synergize to destabilize mitochondria (13) suggest that in oocytes external stresses of both developmental and pathological origins are relayed via ASMase-generated ceramide to a central apoptosis regulatory step governed by Bax.

Another protein that can function upstream of Bax in oocytes is the aromatic hydrocarbon receptor (Ahr), a member of the Per-Arnt-Sim family of transcription factors (14, 15). Recent studies have shown that the Ahr is functionally required for oocytes to die after exposure to PAH (8, 9), a class of ubiquitous environmental contaminants produced primarily as a by-product of fossil fuel combustion that are known to bind and activate the Ahr (14, 15). In both mouse and human ovaries, the PAH-activated Ahr kills oocytes by increasing *bax* gene expression (8, 9), a key step in the cytotoxic response, given that Bax-deficient oocytes fail to undergo apoptosis when exposed to PAH either *in vitro* (8) or *in vivo* (9). Interestingly, a loss of Ahr function in mice in the absence of chemical exposure reduces the incidence of fetal oocyte death, leading to the birth of female offspring with 2-fold more primordial follicles in their ovarian reserves than normal (16). Therefore, it may be that oocytes lacking this transcription factor are inherently more resistant to apoptosis regardless of stimulus, due to changes in gene expression patterns resulting from chronic Ahr deficiency.

Analyses of mice lacking caspase-2, a member of a family of related cysteine proteases that produce the morphological and biochemical features of apoptotic cells (17), have revealed its importance to developmental and chemotherapy-induced apoptosis in oocytes (10, 18). In addition, results from both gene expression (19) and biochemical (20) studies have implicated caspase-3, another key member of this apoptotic protease family (17), in the mediation of oocyte apoptosis. However, recent experiments using caspase-3-deficient mice demonstrated that this enzyme, although critical for ovarian granulosa (21) and luteal (22) cell death, is not needed for oocytes to die in response to either developmental cues or anticancer drugs (21). Whether caspase-3 is functionally required for oocytes to execute apoptosis in response to other cytotoxic stimuli is currently unknown.

This latter point is of particular interest in light of data showing that increases in caspase-3 protein, processing, and activity occur in oocytes and granulosa cells of immature follicles in ovaries of rats exposed to the toxic environmental chemical, 4-vinylcyclohexene diepoxide (VCD) (23). Previous work established that VCD, a by-product of the manufacture of plastics, rubber, flame retardants, and pesticides, causes depletion of immature (primordial and primary) follicles from the ovaries of rats and mice (24). Furthermore, apoptosis is involved as a mediator of follicle loss caused by VCD exposure (25), and several Bcl-2 family members have been identified as potential modulators of this paradigm of follicle atresia (26, 27). Therefore, a molecular cell death pathway involving specifically Bax, as a key regulator, and caspase-3, as a key executioner, has been proposed as the underlying mechanism responsible for VCD-induced ovarian failure (23–27). Accordingly, we designed the present experiments to test the functional importance of both Bax and caspase-3 as well as of ASMase, the Ahr, and caspase-2 in primordial and primary follicle depletion resulting from VCD exposure *in vivo*.

Materials and Methods

Animals

Heterozygous *Ahr* ($Ahr^{+/-}$) male and female mice, obtained from The Jackson Laboratory (Bar Harbor, ME; C57BL/6-Ahr^{tm1Bra}), were used to generate wild-type and Ahr-deficient female littermates. Studies describing the generation or genotyping of mice lacking ASMase (28), the Ahr (16), Bax (29), caspase-2 (18), and caspase-3 (30) have been reported previously. For each mutant mouse line, wild-type and homozygous-null female mice, derived from mating of heterozygous males and females, were used for experimentation. However, for the *Ahr* mutant studies, two additional C57BL/6 wild-type female mice were purchased (The Jackson Laboratory) and used for either vehicle or VCD injections to increase the sample size in these two treatment groups from three to four. The Ahr-, Bax-, and caspase-3-deficient mouse lines were C57BL/6 congenic (*i.e.* more than nine generations), whereas the ASMase- and caspase-2-deficient mouse lines were of a mixed C57BL/6–129/Sv background. All animal protocols were reviewed and approved by the institutional animal care and use committees of Massachusetts General Hospital and Boston University School of Medicine and were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

VCD dosing regimen

Following a protocol routinely used to study the ovotoxic effects of VCD *in vivo* (23–27), female mice were given once-daily ip injections of either vehicle (sesame oil) or VCD (80 mg/kg body weight; Pfaltz and Bauer, Inc., Waterbury, CT) for 15 d, starting on d 27 (*ASMase*, *bax*, *caspase-2*, and *caspase-3*) or 35 (*Ahr*) postpartum. Ovaries were collected 24 h after the final injection for histomorphometric analysis of follicle numbers, as described below.

Follicle counts

Ovaries were fixed (0.34 N glacial acetic acid, 10% formalin, and 28% ethanol), embedded in paraffin, and serially sectioned (8 μm). The serial sections from each ovary were aligned in order on glass microscope slides, stained with hematoxylin and picric methyl blue, and analyzed for the number of nonatretic primordial and primary follicles in every fifth section with random start, as detailed previously (5, 31). As this procedure samples one fifth of the entire ovarian volume, the total number of follicles per ovary was then estimated by multiplying the cumulative counts for each ovary by a correction factor of 5 (*i.e.* the total number of ovarian sections divided by the total number of ovarian sections counted).

Data presentation and statistical analysis

Four of the five mutant mouse lines used in this study (ASMase-, Ahr-, Bax- and caspase-2-deficient) exhibit alterations in the total number of immature follicles per ovary due to the gene inactivation (5, 12, 16, 18, 21). Therefore, the effect of VCD on follicle number was calculated for each genotype group (*ASMase*-null, *Ahr*-null, *bax*-null, *caspase-2*-null, and *caspase-3*-null along with their respective wild-type female siblings) as a percentage of primordial or primary follicles destroyed *vs.* the respective vehicle-treated control (wild-type or gene mutant). The data shown represent the mean ± SEM of the combined results from the analysis of ovaries collected from three or more mice per genotype per treatment group. A *t* test was used for comparison of mean values obtained when assessing the effect of genotype on the percentage of follicles destroyed by VCD treatment, and $P < 0.05$ was chosen to indicate a statistically significant difference.

Results

As the various lines of mutant mice used herein differed slightly with respect to genetic background (C57BL/6 con-

genic or a mix of C57BL/6 and 129/Sv), we initially tested whether these differences in genetic strain had any discernible effect on the extent of follicle destruction caused by VCD exposure. Wild-type female mice derived from the five separate colonies of heterozygous breeding animals were treated with VCD for 15 d and analyzed for oocyte number. No significant differences in the extent of primordial follicle destruction were noted among the various groups (Table 1). However, the extent of primary follicle destruction caused by VCD treatment differed slightly in wild-type mice derived from the *bax* mutant *vs.* the *caspase-3* mutant colony (Table 1).

We then examined the impact of the indicated gene knockouts on the extent of VCD-induced follicle destruction. From the first series of these experiments we observed that ovaries collected from wild-type and ASMase-deficient female siblings, treated in parallel for 15 d with VCD, showed no significant differences in the levels of primordial or primary follicle destruction (Fig. 1). Similar findings were observed from studies of *Ahr*-null mice in that the gene knockout had no significant effect on the extent of primordial and primary follicle loss resulting from VCD treatment (Fig. 2). In contrast, the extent of VCD-induced primordial follicle depletion in Bax-deficient mice was significantly ($P < 0.05$) lower than that in wild-type females (Fig. 3). The extent of primary follicle loss in Bax-deficient mice exposed to VCD was also significantly ($P < 0.05$) lower than that in wild-type controls (Fig. 3).

From the fourth series of experiments we observed that significantly ($P < 0.05$) fewer oocyte-containing primary follicles were destroyed by VCD in caspase-2-deficient mice compared with wild-type controls (Fig. 4). However, no significant difference in the extent of VCD-induced primordial follicle destruction was observed in caspase-2-deficient *vs.* wild-type females (Fig. 4). Finally, we found that significantly ($P < 0.05$) fewer oocyte-containing primary follicles were destroyed by VCD in caspase-3-deficient mice compared with wild-type controls (Fig. 5). However, no significant difference in the extent of VCD-induced primordial follicle loss was observed in caspase-3-deficient *vs.* wild-type females (Fig. 5).

TABLE 1. Comparison of VCD-induced primordial (*upper*) and primary (*lower*) follicle destruction in wild-type female mice on a C57BL/6 congenic (*Ahr, bax, caspase-3*) or a mixed C57BL/6-129/Sv (*ASMase, caspase-2*) background

Mouse line	Primordial follicles, vehicle	Primordial follicles, VCD	Primordial follicle destruction (%)
ASMase	2295 ± 421	430 ± 84	81 ± 3[a]
Ahr	985 ± 71	181 ± 36	82 ± 4[a]
bax	1270 ± 189	190 ± 26	85 ± 2[a]
caspase-2	1162 ± 268	239 ± 74	79 ± 6[a]
caspase-3	2294 ± 526	468 ± 68	80 ± 3[a]
Mouse line	**Primary follicles, vehicle**	**Primary follicles, VCD**	**Primary follicle destruction (%)**
ASMase	509 ± 116	106 ± 40	79 ± 6[a,b]
Ahr	311 ± 24	54 ± 19	83 ± 6[a,b]
bax	131 ± 18	19 ± 5	86 ± 4[a]
caspase-2	183 ± 64	53 ± 11	71 ± 6[a,b]
caspase-3	283 ± 24	81 ± 7	71 ± 2[b]

Values are the mean ± SEM of combined data from the analysis of four mice per group.

[a,b] Values w*ithin the last column with different superscript letters*, $P < 0.05$.

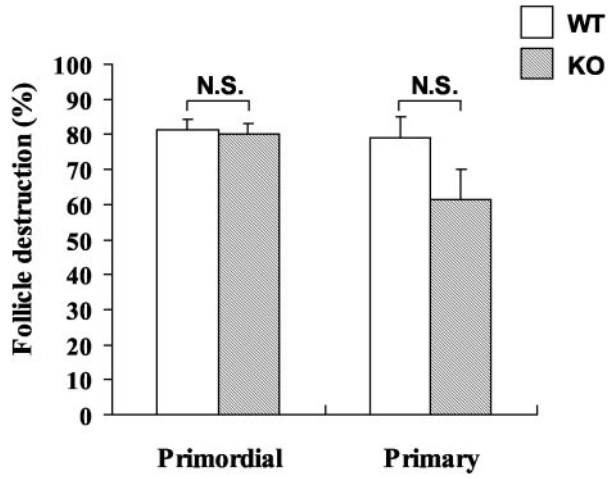

FIG. 1. ASMase deficiency has no effect on the extent of VCD-induced follicle destruction. Values represent the percentage of primordial or primary follicles destroyed by VCD, compared with vehicle-treated controls, in wild-type (WT) and *ASMase*-null [knockout (KO)] female mice treated in parallel (mean ± SEM; n = 4 mice/group). N.S., Not significant.

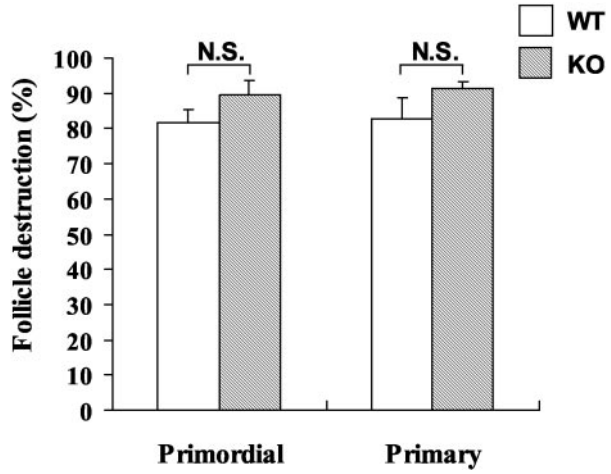

FIG. 2. The Ahr is not required for VCD to kill follicles. Values represent the percentage of primordial or primary follicles destroyed by VCD, compared with vehicle-treated controls, in wild-type (WT) and *Ahr*-null [knockout (KO)] female mice treated in parallel (mean ± SEM; n = 4 mice/group). N.S., Not significant.

Discussion

In the present studies we evaluated the requirement of five gene products, proposed by ourselves and others as being involved in female germ cell death and immature follicle atresia, for VCD-induced depletion of primordial and primary follicles in the mouse ovary. The first gene product, ASMase, was selected for analysis because previous work has established that this ceramide-generating enzyme is required for the death of oocytes under normal conditions as well as after exposure to a chemotherapeutic drug (12). Fur-

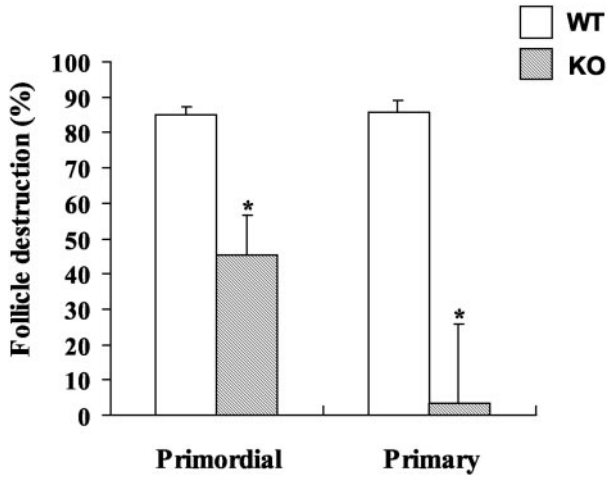

FIG. 3. Loss of Bax function protects primordial and primary follicles from VCD-induced destruction. Values represent the percentage of primordial or primary follicles destroyed by VCD, compared with vehicle-treated controls, in wild-type (WT) and *bax*-null [knockout (KO)] female mice treated in parallel (mean ± SEM; n = 4 mice/group). *, $P < 0.05$ *vs.* respective WT value.

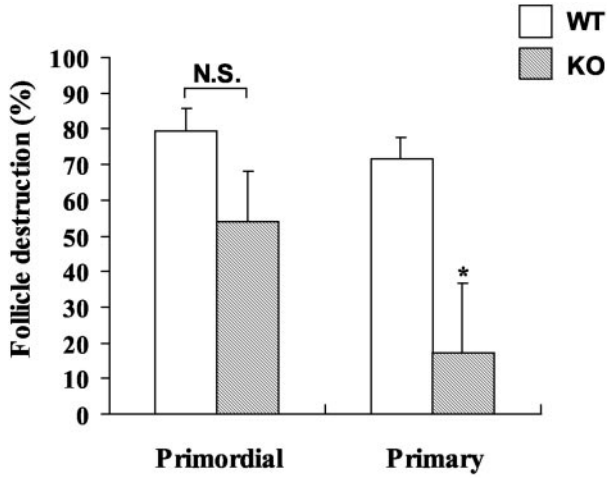

FIG. 4. Caspase-2 deficiency partially protects primary, but not primordial, follicles from the cytotoxic effects of VCD. Values represent the percentage of primordial or primary follicles destroyed by VCD, compared with vehicle-treated controls, in wild-type (WT) and *caspase-2*-null [knockout (KO)] female mice treated in parallel (mean ± SEM; n = 4 mice/group). *, $P < 0.05$ *vs.* respective WT value; N.S., not significant.

thermore, VCD is known to trigger an oxidative stress response in immature follicles of the rat ovary (26). Given that oxidative stress has been identified as a stimulus for ceramide generation leading to apoptosis (32), one could logically envisage that the initial cellular response to VCD exposure involves the production of ceramide as a stress sensor. Results from treating ASMase-deficient mice with VCD, however, did not support this proposal, because we observed no protective effect of this gene knockout on follicle survival. Although these findings argue against a critical role for ASMase in VCD-induced ovotoxicity, it is important to point out that ceramide can be generated by more than one mechanism (11). Thus, we cannot rule out the involvement of ceramide as a mediator of VCD-induced ovarian failure.

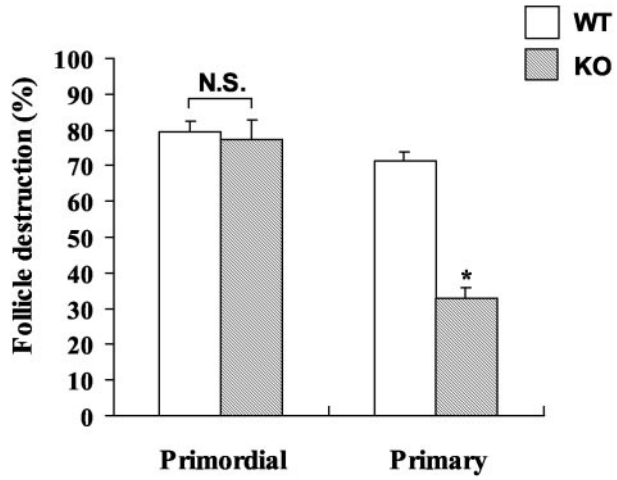

FIG. 5. Absence of caspase-3 partially protects primary, but not primordial, follicles from the cytotoxic effects of VCD. Values represent the percentage of primordial or primary follicles destroyed by VCD, compared with vehicle-treated controls, in wild-type (WT) and *caspase-3*-null [knockout (KO)] female mice dosed in parallel (mean ± SEM; n = 3 or 4 mice/group). *, $P < 0.05$ *vs.* respective WT value; N.S., not significant.

Future work, possibly employing the use of the ceramide inhibitor sphingosine-1-phosphate as recently described for protecting the ovaries from radiation-induced damage (12, 33), may provide additional insight into the requirement, if any, of ceramide in this model of follicle loss.

In the second series of experiments we tested whether Ahr deficiency altered the extent of follicle death caused by VCD treatment. These experiments were designed in light of previous studies demonstrating that this receptor for PAH and halogenated biphenyls (*e.g.* 2,3,7,8-tetrachlorodibenzo-*p*-dioxin) is required for PAH to destroy primordial and primary follicles in the mouse ovary (8). In addition, Ahr loss of function in the absence of PAH exposure leads to enhanced oocyte survival during fetal ovarian development (16), suggesting that this transcription factor serves as a modulator of oocyte death susceptibility under diverse conditions. However, like ASMase deficiency, a lack of functional Ahr was not found to alter the ovotoxic response to VCD treatment. These findings not only rule out a role for the Ahr in this model of follicle depletion, but also underscore the specificity of Ahr function in oocyte death induced by as yet unknown developmental cues (16) as well as by PAH exposure (8, 9). This latter point provides yet another example supportive of the concept that different stimuli recruit genetically distinct pathways to signal oocyte death (2), although there may still be a common convergence point somewhere downstream that is central to germ cell depletion and ovarian failure.

In this regard the next gene product analyzed was Bax, a proapoptotic member of the Bcl-2 family of programmed cell death regulators (3, 4). Bax was a logical candidate for analysis based on a number of reports that this protein is required for the initiation of oocyte death under diverse conditions (5–9). Furthermore, past studies have shown that immature follicles in the ovaries of rats exposed to VCD exhibit elevated levels of *bax* mRNA (26) as well as a redistribution of

Bax protein from the cytosolic to the mitochondrial compartment (27). In keeping with the theme of these studies and in contrast to the results with ASMase- and Ahr-deficient mice, we observed that Bax loss of function conveyed significant protection against the cytotoxic effects of VCD in both primordial and primary follicles. Therefore, we can add VCD to the long list of proapoptotic agents and stimuli that Bax deficiency can protect oocytes from (1, 2). However, it should be noted that the protective effects of Bax deficiency were partial, as primordial follicle destruction still occurred after VCD exposure, albeit at reduced levels compared with wild-type female siblings treated in parallel. Therefore, these data suggest the existence of additional cell death mechanisms that function independently of Bax as being at least partly responsible for VCD-induced ovotoxicity.

In the final series of experiments we explored the potential requirement of two different caspase family members for ovarian follicle depletion resulting from VCD treatment. The first enzyme analyzed was caspase-2, a protease previously identified as being needed for oocyte death both during fetal ovarian development and after anticancer drug exposure (10, 15). Similar to Bax-deficient mice, mice lacking caspase-2 showed a reduced level of primary follicle depletion after VCD treatment compared with the extent of primary follicle loss observed in VCD-treated wild-type controls. Somewhat surprisingly, however, caspase-2 deficiency conveyed no significant protective effect against VCD-induced toxicity in the primordial follicle pool, even with three more animals added to increase the sample size from four to seven (data not shown). The reason(s) for this remains unclear, although we initially assumed that another caspase family member could be involved in executing follicular cell death caused by VCD. In this regard, recent work has proposed a central role for caspase-3 as being central to the cytotoxic effects of VCD in the rat ovary (23). Therefore, we next tested whether *caspase-3* gene knockout altered the ovotoxic response to VCD. Results from these experiments revealed that this caspase family member, like caspase-2, was partly involved in VCD-induced primary, but not primordial, follicle atresia. These data, which support and extend correlative evidence from studies of rats implicating caspase-3 as a major player in this paradigm of follicle loss (23), indicate that VCD activates a cell death pathway in primary follicles that requires both caspase-2 and caspase-3 for its execution.

At present, we are unsure why primordial follicles are not protected from VCD-induced death in caspase-2- or caspase-3-deficient mice, especially since the loss of Bax function is protective in this follicle pool. Some insight may come from the reported ovarian phenotypes of caspase-2- and caspase-3-deficient mice, which exhibit cell lineage-selective, if not specific, defects in apoptosis (10, 18, 21), *vs. bax*-null females, which show enhanced survival of both granulosa cells (29) (Korsmeyer, S. J., and J. L. Tilly, unpublished data) and oocytes (5–9). In contrast to either *caspase* gene knockout, Bax deficiency protected both primordial and primary follicles from the cytotoxic effects of VCD. Given that caspase-2 is critical for oocyte death (10, 18), whereas caspase-3 is needed for granulosa cell apoptosis (21), a double-mutant female mouse lacking both executioner enzymes may respond to VCD treatment with a reduction in the extent of primordial follicle loss. Although beyond the scope of the present studies, experiments have been initiated to generate sufficient numbers of *caspase-2/caspase-3* double-mutant female mice to eventually test this hypothesis.

In closing, tremendous progress has been made in recent years toward elucidating the cellular and molecular mechanisms responsible for mediating oocyte death and follicle depletion under both normal and pathological conditions (2). Numerous studies have now collectively underscored the importance of Bax and caspase-2 to multiple paradigms of oocyte and follicular cell apoptosis (5–10, 18, 29), including VCD-induced ovotoxicity as shown herein. However, work in this field has also revealed that the involvement of other proteins implicated in oocyte or follicle death is stimulus specific [ASMase (Ref. 12 and present studies), Ahr (Refs. 8, 9, and 16 and present studies), caspase-3 (Ref. 21 and present studies)]. Moreover, it is important to keep in mind that although Bax has emerged as a central component of the oocyte death program under many conditions (Refs. 5–9 and present studies), the protein is not needed for all paradigms of oocyte loss (10). Therefore, in addition to confirming that Bax, caspase-2, and caspase-3 are functionally important mediators of VCD-induced ovotoxicity, the present study supports the concept that the specific pathway used by follicles to die is determined by both the developmental status of the oocyte and the stimulus responsible for apoptosis (2).

Acknowledgments

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