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# Inhibition of Ovarian KIT Phosphorylation by the Ovotoxicant 4-Vinylcyclohexene Diepoxide in Rats[1]

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## ABSTRACT

**In vitro exposure of Postnatal Day 4 (PND4) rat ovaries to the occupational chemical 4-vinylcyclohexene diepoxide (VCD) destroys specifically primordial and primary follicles via acceleration of atresia. Because oocyte-expressed c-kit (KIT) plays a critical role in follicle survival and activation, a direct interaction of VCD with KIT as its mechanism of ovotoxicity was investigated. PND4 rat ovaries were cultured with and without VCD (30 μM) for 2 days. When assessed by Western analysis or mobility shift detection, phosphorylated KIT (pKIT) was decreased ($P < 0.05$) by VCD exposure, while total KIT protein was unaffected. Anti-mouse KIT2 (ACK2) antibody binds KIT and blocks its signaling pathways, whereas anti-mouse KIT 4 (ACK4) antibody binds KIT but does not block its activity. PND4 rat ovaries were incubated for 2 days with and without VCD with and without ACK2 (80 μg/ml) or ACK4 (80 μg/ml). ACK2 decreased pKIT; however, ACK4 had no effect. Conversely, ACK2 did not affect a VCD-induced decrease in pKIT, whereas ACK4 further reduced it. Because ACK2 and ACK4 (known to directly bind KIT) affect VCD responses, these results support the fact that VCD interacts directly with KIT. The effect of these antibodies on VCD-induced follicle loss was measured after 8 days of incubation. ACK2 further reduced ($P < 0.05$) VCD-induced follicle loss, whereas ACK4 did not affect it. These findings demonstrate that VCD induces ovotoxicity by direct inhibition of KIT autophosphorylation of the oocyte. The data also further support the vital function of KIT and its signaling pathway in primordial follicle survival and activation, as well as its role in VCD-induced ovotoxicity.**

*follicle, oocyte–follicle interactions, ovary, signal transduction, toxicology*

## INTRODUCTION

The growth factor KITLG (kit ligand or stem cell factor) and its receptor, KIT, have received considerable attention recently for their indispensible roles in mammalian oogenesis and folliculogenesis. The type III tyrosine kinase receptor KIT is expressed in oocyte and theca cells, while KITLG is expressed in granulosa cells [1, 2]. Binding of KITLG to KIT leads to receptor dimerization and activation of protein kinase activity [3]. The receptor becomes autophosphorylated at tyrosine residues during activation, which is followed by a series of cellular responses via signal transduction cascades.

KITLG and KIT interaction plays an important role in the communication between the oocyte and surrounding granulosa cells. When neonatal mice of various ages were injected with the KIT-blocking antibody anti-mouse KIT2 (ACK2), the result was a disruption in primordial follicle development and primary follicle growth, whereas there was no effect on antral follicle development, ovulation, or lutienization of the ovulated follicle [4]. Thus, signaling between KIT and KITLG appears to be important for growth of the ovarian preantral follicle. This occurs at a stage of development when functional follicle-stimulating hormone receptors are not expressed in the ovary [4, 5]. Therefore, the interaction between KITLG and KIT is vital for the earliest stages of follicular development when input from pituitary gonadotropins via the follicle-stimulating hormone receptor is not yet possible.

The mammalian ovary contains a finite pool of primordial follicles, which continues to decrease during the reproductive lifespan as a consequence of ongoing atresia and ovulation [6]. Once the primordial follicle pool is depleted, the result is cessation of ovulation and onset of reproductive senescence. As a result, any chemical that targets and destroys the primordial follicle pool can lead to premature ovarian failure (early menopause in women). 4-Vinylcyclohexene diepoxide (VCD) is a by-product of the chemical synthesis of rubber tires, insecticides, flame retardants, and plasticizers [7]. VCD is ovotoxic and selectively destroys small preantral (primordial and small primary) follicles in the ovaries of rats and mice via accelerated atresia (apoptosis) [8, 9].

Several studies have investigated ovarian signaling pathways associated with VCD-induced ovotoxicity. Incubation of Postnatal Day 4 (PND4) rat ovaries (highly enriched in primordial and primary follicles) with VCD (30 μM) resulted in follicle loss by Day (D)6 of culture [10]. This in vitro exposure to VCD also caused a decrease in mRNA encoding the *Kit* gene (D4) and an increase in mRNA encoding the *Kitlg* gene (D6), relative to those of control [11]. The increase in KITLG expression was proposed to reflect a granulosa cell feedback response to inhibited oocyte KIT. The specificity of the KITLG/KIT pathway was suggested because co-incubation of exogenous growth and differentiation 9 (GDF9) and bone morphogenic protein 4 (BMP4) with VCD had no effect on VCD-induced ovotoxicity, whereas exogenous KITLG attenuated VCD-induced follicle loss [11]. These growth factors were investigated because of the ability of GDF9 to promote development of early primordial follicles [12] and of BMP4 to

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TABLE 1. The effect of exogenous growth factors on VCD-induced ovotoxicity.

| Growth factor | Growth factor conc. (ng/ml) + VCD (30 μM)[a] | Primordial follicle counts (% of control) | Site of in vivo secretion[d] | Target of growth factor [references][e] |
|---|---|---|---|---|
| Control | 0 | 100.0 ± 5.0 | | |
| VCD | 30 | 27.0 ± 2.5[b] | | |
| KITLG | 50 | 39.4 ± 3.7[b] | Granulosa cells | Stroma and oocyte [5, 18] |
| | 400 | 68.9 ± 1.3[b,c] | | |
| | 800 | 61.5 ± 9.1[b,c] | | |
| LIF | 50 | 47.8 ± 10.3[b] | Granulosa cells | Granulosa cells and oocyte [19] |
| | 400 | 50.0 ± 16.3[b] | | |
| | 800 | 20.3 ± 1.9[b] | | |
| GDNF | 50 | 38.0 ± 5.7[b] | Oocyte | Oocyte [20] |
| | 400 | 24.3 ± 2.3[b] | | |
| | 800 | 30.1 ± 4.8[b] | | |
| PDGFB | 50 | 15.8 ± 3.5[b] | Oocyte | Granulosa and thecal cells [21] |
| | 400 | 15.5 ± 2.4[b] | | |
| | 800 | 9.5 ± 2.1[b] | | |
| FGF2 | 50 | 19.5 ± 6.4[b] | Oocyte | Granulosa and thecal cells [22, 23] |
| | 400 | 11.8 ± 1.3[b] | | |
| | 800 | 18.7 ± 1.9[b] | | |
| FGF7 | 50 | 49.2 ± 8.4[b] | Thecal cells | Granulosa cells [24] |
| | 400 | 42.5 ± 5.4[b] | | |
| | 800 | 48.7 ± 3.9[b] | | |

[a] All values are ng/ml except VCD (μM).
[b] $P < 0.05$ different from control.
[c] $P < 0.05$ different from VCD.
[d] Cellular sites of secretion.
[e] Cellular target.

promote primordial follicle survival and development of early primary follicles [13].

Studies examining downstream members of the KIT/KITLG signaling pathway and its role in VCD-induced ovotoxicity have also been conducted. The PI3 kinase pathway can be activated by KIT and plays an important role in oocyte survival signaling [14]. PI3 kinase inhibition using LY294002 provided primordial follicle protection but enhanced primary follicle loss during VCD-induced ovotoxicity [10]. This observation supported the enhancement by VCD of primordial-to-primary follicle activation/recruitment. VCD has also been shown to inhibit phosphorylation and nuclear localization of AKT (downstream in the KIT pathway) in the oocyte of primordial and primary follicles on D2 of exposure [15]. This highlights the importance of early cell signaling events triggered during VCD-induced ovotoxicity.

Taken together, these previous studies support the hypothesis that VCD impacts the KIT receptor signaling pathway in the oocyte for its destructive effects on primordial and primary follicles. Therefore, the present study was designed to investigate the possibility that KIT is directly targeted by VCD as the mechanism of ovotoxicity in those small preantral follicles.

## MATERIALS AND METHODS

### Reagents

VCD (Chemical Abstract Service no. 106-87-6; >99% purity), bovine serum albumin (BSA), ascorbic acid (vitamin C), transferrin, and $MnCl_2$ were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Dulbecco modified Eagle medium nutrient mixture with F-12 (Ham) 1× medium (DMEM-Ham F12), albumin, penicillin/streptomycin (5000 U/ml/5000 μg/ml, respectively), and Hanks balanced salt solution (without $CaCl_2$, $MgCl_2$, or $MgSO_4$) were obtained from Invitrogen (Carlsbad, CA). Millicell-CM filter inserts were purchased from Millipore (Bedford, MA), and 48-well cell culture plates were obtained from Corning Inc. (Corning, NY). Anti-β-actin (ACTB) antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-KIT antibody was purchased from Dako North America, Inc. (Carpinteria, CA). Anti-phosphorylated-KIT (pKIT) antibody was purchased from Cell Signaling Technology (Danvers, MA). Goat anti-mouse and goat anti-rabbit secondary antibodies and BCA (bicinchoninic acid) protein quantification kits were obtained from Pierce Biotechnology (Rockford, IL). ACK2 was purchased from eBioscience (San Diego, CA). Anti-KIT4 antibody (ACK4) was a generous gift from Dr. Minetaro Ogawa at Kumamoto University, Kumamoto, Japan. Phos-tag acrylamide was obtained from the NARD Institute (Osaka, Japan). Recombinant mouse KITLG, recombinant rat platelet-derived growth factor, B polypeptide (PDGFB), recombinant human leukemia inhibitory factor (LIF), recombinant rat basic fibroblast growth factor 2 (FGF2), recombinant mouse keratinocyte growth factor or fibroblast growth factor 7 (FGF7), and recombinant rat glial cell line-derived neurotrophic factor (GDNF) were purchased from R&D Systems (Minneapolis, MN).

### Animals and Neonatal Ovary Collection

A breeding colony was established from Fischer 344 rats originally purchased from Harlan Laboratories (Indianapolis, IN) to use as a source of PND4 female rat pup ovaries for culture. All pregnant animals were housed singly in plastic cages and maintained in a controlled environment (22 ± 2°C; 12L:12D cycle). The animals were provided a standard diet with ad libitum access to food and water and allowed to give birth. All animal experiments were approved by the University of Arizona's Institutional Animal Care and Use Committee. Neonatal PND4 rats were euthanized by $CO_2$ inhalation followed by decapitation. The ovaries were removed and processed for in vitro culture, histological evaluation, or protein isolation as described below.

### In Vitro Ovarian Culture

Ovaries from PND4 F344 rats were cultured as described by Devine et al. [16]. Each ovary was removed, and, after oviduct and excess tissue were trimmed, it was placed on a piece of Millicell-CM membrane floating on 250 μl of DMEM-Ham F12 medium containing 1 mg/ml BSA, 1 mg/ml albumin, 50 μg/ml ascorbic acid, 5 U/ml penicillin/5 μg/ml streptomycin, and 27.5 μg/ml transferrin per well in a 48-well plate previously equilibrated to 37°C. A fine forceps was used to cover the top of the ovary with a drop of medium to prevent drying. Plates containing ovaries were cultured at 37°C and 5% $CO_2$ in air. For those cultures lasting more than 2 days, medium was removed, and fresh medium with or without treatment was replaced every 2 days. Ovaries were treated with vehicle control medium or VCD (30 μM) for 1, 2, or 8 days. The 8-day time point was used for evaluation of follicle loss, as 6 days of culture showed the first evidence of VCD-induced ovotoxicity. Thus, concentrations and conditions of VCD exposure were previously determined [11].

For the growth factor studies, PND4 ovaries were treated with vehicle control medium or VCD (30 μM) with or without growth factors (KITLG, PDGFB), LIF, FGF2, keratinocyte growth factor, or FGF7, and GDNF) at three

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concentrations (50, 400, and 800 ng/ml) for 8 days. All cultured ovaries remained viable up through 8 days in culture, consistent with a previous report [16]. Primordial follicle counts were reported as percentages of control to account for interexperimental variations in total follicles counted in the numerous cultures involved in this experiment (Table 1).

For the anti-mouse ACK2 and anti-mouse ACK4 studies, PND4 ovaries were cultured in control medium with or without ACK2 (80 μg/ml) or ACK4 (80 μg/ml). For the short-term ACK2/ACK4 studies, ovaries were preincubated with ACK2 or ACK4 for 4 h to allow ample time for antibody binding to KIT. Then, VCD (30 μM) was added to the medium of half of the ovaries, and they were cultured for an additional 2 days. Ovaries were homogenized and processed by Western analysis. For a long-term study, another group of ovaries was cultured (8 days) for histological evaluation with or without VCD (30 μM) with or without ACK2 (80 μg/ml) or ACK4 (80 μg/ml) to evaluate the effect on follicle numbers. Dose response studies were completed for ACK2. The 80 μg/ml dose was chosen because it demonstrated optimum decreases in follicular numbers, with no observed toxicity to ovarian tissue (data not shown). The concentration of ACK4 was assigned based on that determined for ACK2.

### Histological Evaluation of Follicle Numbers

Following incubation, cultured ovaries (n = 5/group) were placed in Bouin fixative for 1.5 h, transferred to 70% ethanol, embedded in paraffin, and serially sectioned (5 μm thick), and every sixth section was mounted. All ovarian sections were stained with hematoxylin and eosin. Investigator was blinded to treatment group before counting follicles. Healthy oocyte-containing follicles were classified and counted in every 12th serial section (Figs. 4 and 6). Unhealthy follicles were distinguished from healthy follicles by granulosa cell content of pyknotic bodies and intense eosinophilic staining of oocytes [16]. Follicle population was classified according to the method used in a previous study [16]. Briefly, primordial follicles contained the oocyte surrounded by a single layer of squamous-shaped granulosa cells; small primary follicles contained an oocyte surrounded by 3 to 20 cuboidal granulosa cells; large primary follicles contained an oocyte surrounded by greater than 20 cuboidal granulosa cells. Secondary follicles contained an oocyte surrounded by multiple layers of granulosa cells. In cultured neonatal rat ovaries (D2–D8), no follicle development beyond the secondary stage was observed [16]. Histological images were captured with an Olympus IX-70 model inverted microscope.

### Protein Isolation

Pools of protein homogenates were prepared from cultured whole ovaries (10–15 ovaries per pool; three pools per treatment group) via homogenization in tissue lysis buffer, as described previously [16]. Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10 000 rpm for 15 min. Supernatant was divided into aliquots and stored at −80°C until further use. Protein was quantified using a standard BCA assay protocol on a 96-well assay plate. Emission absorbance values were detected with a λ excitation value of 540 nm on a Synergy HT model multidetection microplate reader using KC4 software (Bio-Tek Instruments Inc., Winooski, VT). Protein concentrations were calculated from a BSA protein standard curve.

### Western Blot Analysis

SDS-PAGE (8%) was used to separate proteins in homogenates (20 μg; n = 3) and subsequently transferred onto polyvinylidene fluoride membranes as previously described [11]. Membranes were blocked for 1 h with shaking at 4°C in 5% milk in TRIS-buffered saline with Tween-20 (TTBS). Membranes were then incubated with primary antibody in 5% milk in TTBS overnight at 4°C. Antibody dilutions were 1:200 for KIT, 1:200 for pKIT, and 1:1000 for ACTB. Membranes were washed three times for 10 min each with TTBS. Horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) was added for 1 h at room temperature. Membranes were washed three times for 10 min each in TTBS, followed by a single wash for 10 min in TRIS-buffered saline. Western blots were detected by chemiluminescence (using ECL Plus chemiluminescence detection substrate, GE Healthcare Life Sciences) and exposed to x-ray film. Densitometry of the appropriate bands was performed using LabWorks software from a Bioimaging system (UVP Inc., Upland, CA). Individual treatment values were normalized to beta-actin and expressed as the relative protein intensity.

For the Phos-tag Western blot experiment, 5.0 mM Phos-tag acrylamide and 10 mM $MnCl_2$ were added to a 6% SDS-PAGE separating gel solution prior to polymerization. The rest of the method was completed using the standard Western blot protocol described above.

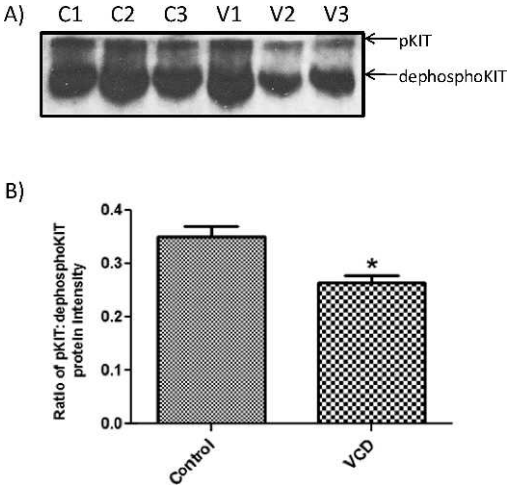



FIG. 1. Effect of VCD on ovarian pKIT protein. Ovaries from PND4 rats were cultured with control medium with or without VCD (30 μM) for 2 days. Phos-tag SDS-PAGE was used to separate pKIT from the dephosphoKIT. **A**) Representative Western blots for KIT expression in cultured ovaries in control medium (C1, C2, C3) or medium containing VCD (V1, V2, V3) are shown. **B**) The ratio of pKIT to dephosphoKIT was calculated and expressed as the mean ± SEM; n = 3; 10 ovaries/pool for cultured ovary samples; $*P < 0.05$ difference from control.

### Statistical Analysis

For experiments with two treatment groups, Student *t*-test was performed. For experiments with two treatment groups and two time points (Fig. 2), a two-way ANOVA with a Bonferroni posttest was performed. For experiments with three or more treatment groups, one-way ANOVA followed by Newman-Keuls posttest was performed. All statistics were calculated with GraphPad Prism version 5.04 software (GraphPad, San Diego, CA). The assigned level of significance for all tests was a $P$ value of $<0.05$.

## RESULTS

### Effect of Ovarian Growth Factors on VCD-Induced Ovotoxicity

A number of exogenous growth factors have been demonstrated to regulate the transition from primordial to primary follicle, including KITLG, LIF, GDNF, PDGFB, FGF1, and keratinocyte growth factor or FGF7. As an approach to demonstrate the specificity of VCD for the KITLG/KIT signaling pathway, we treated PND4 rat ovaries with vehicle control medium or VCD with or without growth factors (KITLG, LIF, GDNF, PDGFB, FGF2, or FGF7) at three concentrations for 8 days (Table 1). In all experiments, VCD treatment reduced the number of primordial follicles ($P < 0.05$) relative to that of control. Addition of LIF, GDNF, PDGFB, FGF2, or FGF7 did not prevent ($P > 0.05$) the VCD-induced decrease in the number of healthy primordial follicles compared to that of control. Conversely, in the presence of exogenous KITLG (400 and 800 ng/ml) there was an attenuation ($P < 0.05$) of VCD-induced follicle loss.

### Effect of VCD Treatment on KIT Phosphorylation in PND4 F344 Rat Ovaries

Time course studies in cultured PND4 F344 rat ovaries report first observing follicle loss on D6 in response to 30 μM VCD [10]. However, variations in subcellular protein distribution of KIT, pAKT, and FOXO3A have been seen as early as D2–D4 after VCD exposure [15]. To further investigate early protein effects, we cultured PND4 F344 rat ovaries with or

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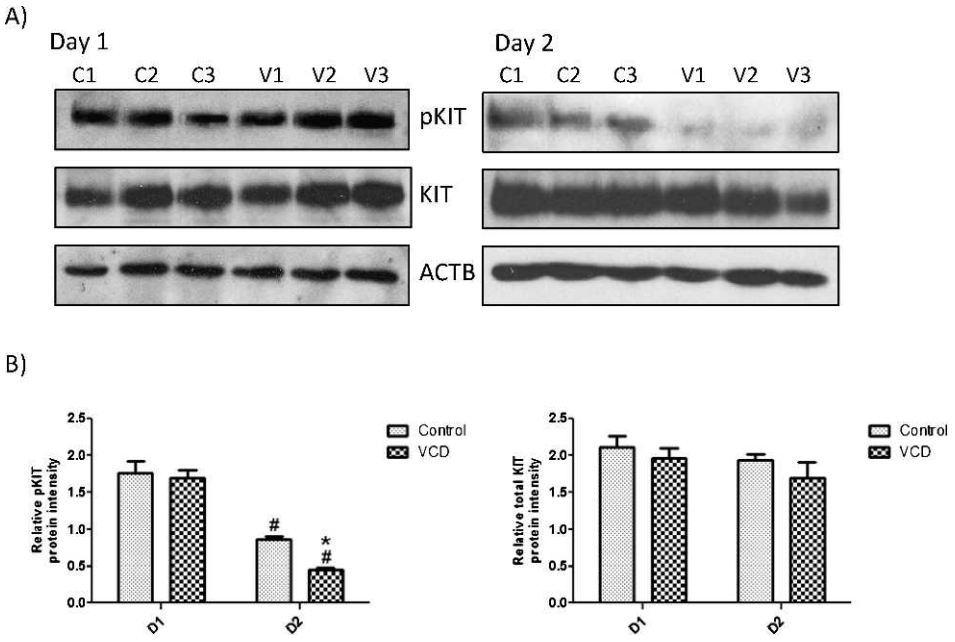



FIG. 2. Effect of VCD on ovarian pKIT for 1 or 2 days exposure is shown. Ovaries from PND4 rats were cultured with control medium with or without VCD (30 μM) for 1 (D1) or 2 (D2) days. **A**) Representative Western blots for pKIT, KIT, and ACTB expression in cultured ovaries exposed to control medium (C1, C2, C3) or medium containing VCD (V1, V2, V3) are shown. **B**) pKIT (left panel) and total KIT (right panel) protein intensity values were normalized to those of ACTB protein and expressed as means ± SEM; n = 3; 10 ovaries/pool for cultured ovary samples; $^{\#}P < 0.05$, D1 vs. D2; $^{*}P < 0.05$, different from respective controls.

without VCD (30 μM) for 1 or 2 days. Two approaches were used to analyze changes in the phosphorylation status of KIT protein following VCD exposure. First, Western blotting following PAGE containing Phos-tag acrylamide-pendant Phos-tag ligand was used. Acrylamide-pendent refers to the ability of the Phos-tag to attach to the acrylamide. When the Phos-tag is attached to the acrylamide in a SDS-PAGE system, it retards the movement of phosphorylated proteins through the gel. This method provides phosphate affinity SDS-PAGE for mobility shift detection of phosphorylated proteins. Relative to protein in control-treated ovaries, pKIT protein was reduced ($P < 0.05$) on D2 of VCD exposure compared to dephosphorylated KIT (dephosphoKIT) protein (Fig. 1, A and B). For the next approach, we used anti-phospho-specific KIT and total anti-KIT antibodies with Western blotting to examine the effect of VCD exposure on pKIT. On D1 there was no effect of VCD on total ovarian pKIT or KIT protein (Fig. 2, A and B). Overall, levels of pKIT were reduced compared to total KIT protein levels ($P < 0.05$) on D2 compared to those on D1. While pKIT protein was decreased ($P < 0.05$) compared to that of control on D2 of VCD exposure, there was no effect of VCD on total KIT protein (Fig. 2, A and B).

### *Effect of KIT Inhibition with ACK2*

To examine the role of VCD in KIT phosphorylation, an ACK2 antibody was used. ACK2 antibody binds to and blocks KIT function [17]. PND4 rat ovaries were preincubated for 4 h

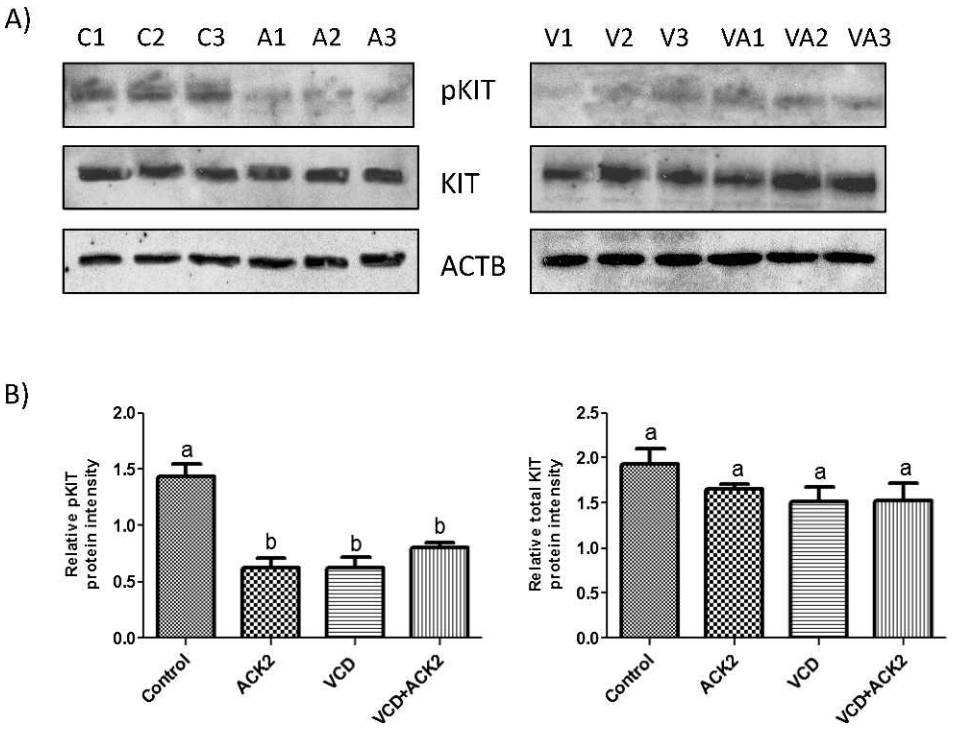



FIG. 3. Effect of ACK2 and VCD on ovarian pKIT is shown. Ovaries from PND4 rats were precultured with control medium with or without ACK2 (80 μg/ml) for 4 h and then VCD (30 μM) was added, or not, to the culture for 2 days. **A**) Representative Western blots for pKIT, KIT, and ACTB expression in cultured ovaries in control medium (C1, C2, C3) or medium containing ACK2 (A1, A2, A3), VCD (V1, V2, V3), or VCD plus ACK2 (VA1, VA2, VA3) are shown. **B**) pKIT (left panel) and total KIT (right panel) protein intensity values were normalized to those of ACTB protein and expressed as means ± SEM; n = 3; 15 ovaries/pool for cultured ovary samples; different superscript letters indicate differences ($P < 0.05$) between the groups.

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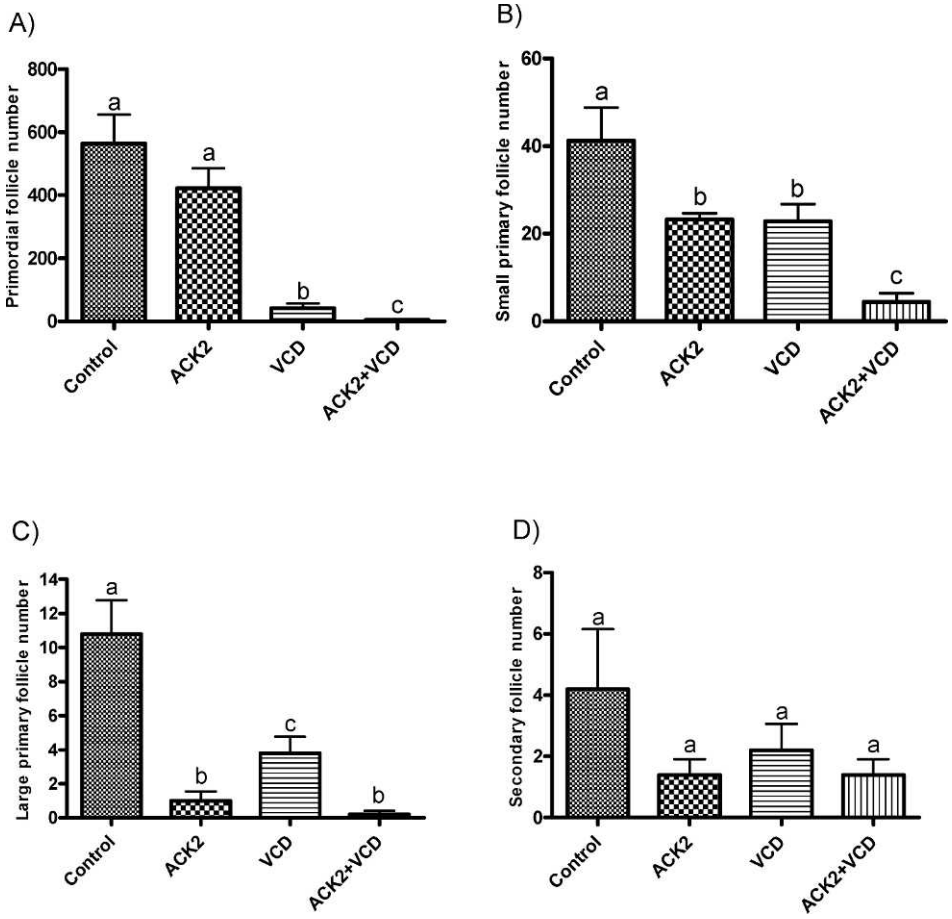



FIG. 4. Effect of ACK2 on VCD-induced follicle loss is shown. PND4 F344 rat ovaries were cultured in culture medium or medium containing VCD (30 μM) with or without ACK2 (80 μg/ml) for 8 days. Ovaries were collected and processed for histological evaluation as described in *Materials and Methods*. Healthy follicles in every 12th section were counted. Values indicate the mean number of healthy primordial follicles (**A**), small primary follicles (**B**), large primary follicles (**C**), and secondary follicles (**D**) counted per ovary ± SEM; n = 5 ovaries per treatment group (**A**–**D**); different superscript letters indicate differences ($P < 0.05$) between the groups.

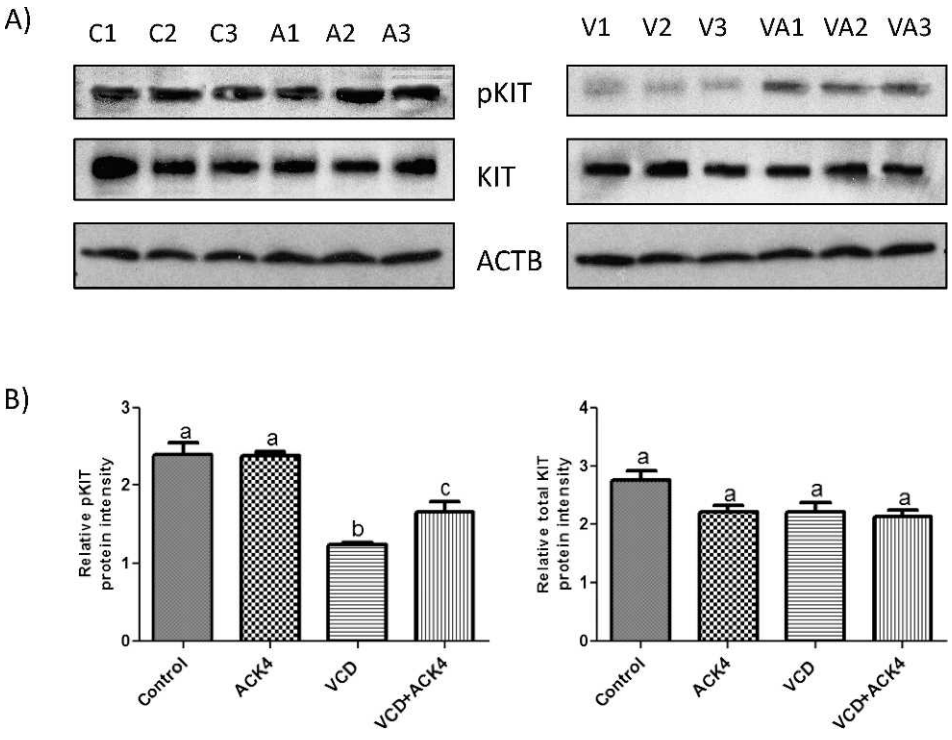



FIG. 5. Effects of ACK4 and VCD on ovarian pKIT are shown. Ovaries from PND4 rats were precultured with control medium with or without ACK4 (80 μg/ml) for 4 h, and then with or without VCD (30 μM) added to the culture for 2 days. **A**) Representative Western blots for pKIT, KIT, and ACTB expression in cultured ovaries in control medium (C1, C2, C3) or medium containing ACK4 (A1, A2, A3), VCD (V1, V2, V3) or VCD plus ACK4 (VA1, VA2, VA3) are shown. **B**) pKIT (left panel) and KIT (right panel) protein intensity values were normalized to those of ACTB protein and expressed as means ± SEM; n = 3; 15 ovaries/pool for cultured ovary samples; different superscript letters indicate differences ($P < 0.05$) between the groups.

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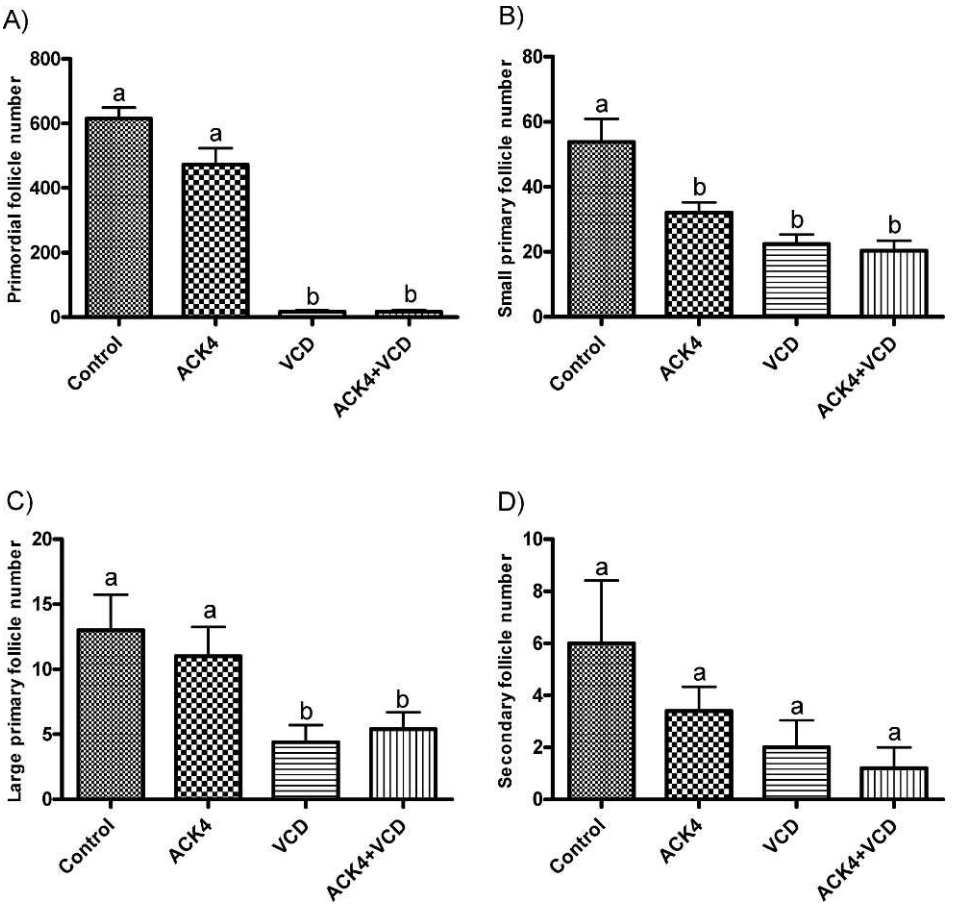



FIG. 6. Effect of ACK4 on VCD-induced follicle loss is shown. PND4 F344 rat ovaries were cultured in culture medium or medium containing VCD (30 μM) with or without ACK4 (80 μg/ml) for 8 days. Ovaries were collected and processed for histological evaluation as described in *Materials and Methods*. Healthy follicles in every 12th section were counted. Values indicate the mean number of healthy primordial follicles (**A**), small primary follicles (**B**), large primary follicles (**C**), and secondary follicles (**D**) counted per ovary ± SEM; n = 5 ovaries per treatment group (**A**–**D**); different superscript letters indicate differences ($P < 0.05$) between the groups.

in control medium or medium containing ACK2 (80 μg/ml) to allow antibody binding, followed by incubation of control medium with or without VCD (30 μM) for an additional 2 days and then analysis by Western blotting. Compared with control, ovarian pKIT protein was reduced ($P < 0.05$) on D2 of ACK2, VCD, and ACK2 plus VCD treatment (Fig. 3, A and B). However, there was no effect of ACK2, VCD, or VCD plus ACK2 treatment on ovarian total KIT protein staining (Fig. 3, A and B).

To examine the effects of disruptions in KIT signaling on VCD-induced ovotoxicity, PND4 Fischer 344 rat ovaries were cultured in control medium or medium containing ACK2 (80 μg/ml) with or without VCD (30 μM) for 8 days. ACK2 treatment reduced ($P < 0.05$) primary (small and large) follicle numbers relative to those of controls but had no effect on primordial follicle numbers (Fig. 4, A, B, and C). ACK2 had no effect on secondary follicles (Fig. 4D). Follicle loss ($P < 0.05$) occurred with VCD in culture in primordial and primary follicles relative to those of control. ACK2 further enhanced ($P < 0.05$) VCD-induced primordial and primary follicle loss.

### *Effect on KIT Binding by ACK4*

Because inhibition of KIT by ACK2 had substantial effects on KIT phosphorylation and follicle numbers, another KIT-specific antibody, ACK4, was utilized to further investigate the role of VCD on KIT phosphorylation. ACK2 and ACK4 recognize different epitopes on the KIT receptor [17]. Whereas ACK2 acts as an antagonistic blocker of KIT signaling function, ACK4 binds to KIT but does not block its activity [17]. PND4 rat ovaries were preincubated for 4 h in control medium or medium containing ACK4 (80 μg/ml) to allow antibody binding, followed by incubation of control medium with or without VCD (30 μM) for an additional 2 days. ACK4 exposure had no effect on pKIT or KIT protein intensity (Fig. 5, A and B). Similar to previous results, VCD caused a decrease in pKIT protein ($P < 0.05$) with no effect on total KIT protein. Preincubation of ovaries with ACK4 attenuated the reduction in pKIT protein seen with VCD (Fig. 5B). There was no effect of ACK4, VCD, or VCD plus ACK4 treatment on total KIT protein (Fig. 5, A and B).

To assess the effect of ACK4 on VCD-induced ovotoxicity, we cultured PND4 Fischer 344 rat ovaries in control medium or medium containing ACK4 (80 μg/ml) with or without VCD (30 μM) for 8 days. ACK4 treatment decreased ($P < 0.05$) small primary follicles relative to those of control but had no effect on primordial or large primary follicle numbers (Fig. 6, A, B, and C). Similar to effects of ACK2, ACK4 had no effect on secondary follicles (Fig. 6D). Whereas ACK2 enhanced VCD-induced small follicle loss, ACK4 did not affect it.

## DISCUSSION

It is well documented that VCD causes ovotoxicity by acceleration of follicular atresia (apoptosis) in primordial and primary follicles [8, 9, 18, 19]. It appears that the effect of VCD on the ovary involves disruption in follicular survival signaling. Previous studies demonstrated that exogenous KITL (50–400 ng/ml) is able to attenuate VCD-induced ovotoxicity [11]. Furthermore, VCD caused a decrease in the *Kit* mRNA expression and an increase in the *Kitl* mRNA expression. VCD has also been shown to impose its ovotoxic effects via inhibition of downstream signaling events in the KITLG/KIT signaling pathway [10, 15]. Taken together, these studies

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support the hypothesis that VCD induces ovotoxicity by directly targeting the oocyte via inhibition of KIT-mediated signaling components.

A variety of ovarian signaling pathways have been shown to be important in folliculogenesis and follicular survival, and these growth factors are released by and have their effects on a variety of follicular compartments. KITLG and LIF are produced by ovarian granulosa cells [5, 20, 21], while GDNF, PDGFB, and FGF2 are produced by the oocyte [22–25]. On the other hand, FGF7 is produced by ovarian theca cells [26]. Previously, whereas exogenous KIT attenuated VCD-induced ovotoxicity, GDF-9 and BMP4 were ineffective at protecting cells from this follicle loss [11]. BMP4 is the only one of those growth factors that has been reported to promote primordial follicle survival [13]. The other growth factors are thought to be involved in primordial-to-primary follicle transition. The present study was initiated to investigate whether other known relevant growth factors play a role in VCD-induced ovotoxicity. Of all growth factors tested, only KITLG was able to attenuate VCD-induced ovotoxicity. This finding further indicates that VCD is acting directly via the KITLG/KIT pathway, rather than by nonspecifically compromising multiple signaling pathways involved in follicle viability or growth.

Because an early event in VCD-induced ovotoxicity appears to be disruption of the KIT signaling pathway, the possibility of direct interaction of VCD with KIT as its mechanism of ovotoxicity in primordial and small primary follicles was investigated. Binding of KITLG to KIT leads to receptor dimerization and activation of protein kinase activity [3]. As a result, the receptor becomes autophosphorylated at tyrosine residues, and activated KIT can then catalyze phosphorylation of downstream substrate proteins. Because phosphorylation status is an important marker of KIT activation, the effect of VCD on pKIT levels was investigated. A phosphorylated protein-specific mobility shift assay of KIT protein was conducted using a Phos-tag acrylamide-pendant Phos-tag ligand. That experiment demonstrated a decrease in pKIT protein with VCD exposure for 2 days. The reduction in pKIT protein on D2 was confirmed using a pKIT-specific antibody. No effect on pKIT was observed on D1 of VCD exposure. Thus, the decrease in pKIT on D2 is the earliest observed evidence of a direct interaction between VCD and KIT. This observation supports the fact that VCD-induced ovotoxicity is initiated by direct interaction of VCD with KIT. However, it is possible that VCD could also interact with a protein upstream of KIT or membrane elements in the vicinity of KIT to change its orientation. Current studies are underway to investigate this possibility. By whatever mechanism, this finding highlights the fact that a change in KIT receptor phosphorylation status is a critical early component of VCD-induced follicle loss.

In vitro exposure of ovaries to ACK2 has been shown to block KIT, inhibit oocyte growth, and induce follicular atresia [27, 28]. Additionally, in vivo exposure to ACK2 has been shown to disrupt the onset of primordial follicle development, primary follicle growth, and follicular fluid formation [4]. These effects of ACK2 on the ovary may be due in part to the reduced phosphorylation of glycogen synthase kinase-3 (a downstream member of the KITLG/KIT signaling pathway) [29]. Collectively, these reports have demonstrated that blocking of KIT function by ACK2 can have a dramatic effect on ovarian preantral follicle development and survival.

Because KIT appears to play a vital role in VCD-induced ovotoxicity, an experiment was conducted to determine if blocking KIT function by ACK2 could affect the inhibition of KIT phosphorylation by VCD. Whereas neither VCD nor ACK2 affected total KIT protein on D2, ACK2 with or without VCD caused a similar decrease in pKIT protein. This supports the presence of a similar mechanism of interaction with KIT between ACK2 and VCD. Because these changes in pKIT were seen on D2 of culture, the effect of ACK2 (and its inhibition of KIT signaling) on VCD-induced ovotoxicity on D8 was investigated. ACK2 decreased small and large primary follicles, indicating the ability to block recruitment of primordial follicles into primary follicles. The reason that increased primordial follicle numbers were not seen in the face of inhibited recruitment is not clear. However, due to the much greater number of primordial follicles than primary follicles, absolute differences in numbers between the two pools would likely be difficult to measure. Co-incubation of VCD and ACK2 further decreased primordial, as well as small and large primary follicles, compared to that of VCD treatment alone. Interestingly, there was no additive effect of ACK2 plus VCD on inhibition of KIT phosphorylation on D2. Therefore, the reason for a greater combined inhibition of follicle numbers is not clear. It could reflect a post-KIT divergence of downstream signaling events within the oocyte. Alternatively, there may be temporal variations between pathways affected by ACK2 and VCD, as observed on D8.

Both ACK2 and ACK4 recognize the extracellular domain of KIT; however, they are directed against different epitopes and, therefore, can have different effects on KIT function. While ACK2 is an effective antagonist of KIT, ACK4 binds to the receptor but does not impair its function in hematopoietic progenitor cells [17]. To our knowledge, the effect of ACK4 on ovarian KIT has not been reported. Therefore, an experiment was conducted to evaluate the effects of ACK4 within the ovary and to compare them with those of ACK2. Furthermore, the effects on VCD-induced ovotoxicity were studied. In vitro incubation of PND4 rat ovaries with ACK4 (D2) had no effect on pKIT protein. Interestingly, coculture of ovaries with ACK4 and VCD attenuated the decreased phosphorylation of KIT protein caused by VCD. Because ovaries were preincubated (4 h) with ACK4 prior to addition of VCD, ACK4 may have partially protected KIT from interactions with VCD. Alternatively, ACK4 binding to KIT may have altered its conformation to interfere with its ability to be affected by VCD. There was no effect of treatment with ACK4, VCD, or VCD plus ACK4 on total KIT protein. As with ACK2, the effect of ACK4 on VCD-induced ovotoxicity was also evaluated. ACK4 exposure decreased small primary follicle numbers, whereas there was no effect on other follicle types. Unlike ACK2, ACK4 did not exacerbate VCD-induced follicle loss. This provides support for the possibility of impairment of similar signaling events downstream of ACK4 and VCD that are involved in primordial follicle activation. ACK2 and ACK4 are known to directly bind KIT, and VCD effects were impacted by those antibodies. Therefore, these results provide further convincing evidence that VCD directly interacts with KIT.

Because neither ACK2 nor ACK4 had an effect on primordial follicles but decreased small primary follicle numbers suggests that they both retard primordial follicle activation and recruitment. However, ACK2 provided a substantial blockage of primordial follicle recruitment, as reflected by the diminished pool of large primary follicles. Conversely, ACK4 had no such effect. These observations support the previously described ability of ACK2 to block primordial follicle recruitment more completely than ACK4 [4]. They also provide the first known report of an ovarian effect of ACK4.

While KIT expression has been demonstrated in other tissues, the high expression of KIT and KITLG during the primordial to the large preantral stages of follicular growth is

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most relevant for explaining VCD's ability to initiate ovotoxicity specifically in primordial and small primary follicles [30, 31]. The primordial follicle pool is finite, so once destroyed or depleted, those follicles cannot be regenerated [6]. Whereas other tissues that express KIT may potentially be targeted by VCD, only oocytes contained in primordial follicles within the ovary are incapable of being replaced with new cells if destroyed. This explains why no long-term effects of in vivo exposure to VCD have been observed in other tissues [32]. Therefore, direct targeting of KIT by VCD can help explain its physiologically specific effect.

In summary, the present study further underscores the role of KIT in primordial follicle survival, activation, and recruitment. Furthermore, these results also provide strong evidence for direct KIT targeting in VCD-induced ovotoxicity. Currently, studies are underway to identify the specific downstream signaling pathways within the oocyte that are involved in primordial follicle survival/recruitment as they are impacted by VCD. Additionally, proteomic approaches are being used to identify a possible binding site of VCD on KIT. These results further showcase posttranslational effects in the KITLG/KIT pathway within the oocyte as the specific target of VCD for induction of ovotoxicity.

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