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Source: Biology of Reproduction, 84(4) : 743-751

Published By: Society for the Study of Reproduction

URL: https://doi.org/10.1095/biolreprod.110.087650

BIOLOGY OF REPRODUCTION **84,** 743–751 (2011)
Published online before print 10 November 2010.
DOI 10.1095/biolreprod.110.087650

Inhibition of PIK3 Signaling Pathway Members by the Ovotoxicant 4-Vinylcyclohexene Diepoxide in Rats[1]

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ABSTRACT

4-Vinylcyclohexene diepoxide (VCD), an occupational chemical that specifically destroys primordial and small primary follicles in the ovaries of rats and mice, is thought to target an oocyte-expressed tyrosine kinase receptor, Kit. This study compared the temporal effect of VCD on protein distribution of KIT and its downstream PIK3-activated proteins, AKT and FOXO3. Postnatal Day 4 Fischer 344 rat ovaries were cultured in control media ± VCD (30 μM) for 2–8 days (d2–d8). KIT, AKT, phosphorylated AKT, FOXO3, and pFOXO3 protein levels were assessed by Western blotting and/or immunofluorescence staining with confocal microscopy. Phosphorylated AKT was decreased ($P < 0.05$) in oocyte nuclei in primordial (39% decrease) and small primary (37% decrease) follicles within 2 days of VCD exposure. After d4, VCD reduced ($P < 0.05$) oocyte staining for KIT (primordial, 44% decrease; small primary, 39% decrease) and FOXO3 (primordial, 40% decrease; small primary, 36% decrease) protein. Total AKT and pFOXO3 were not affected by VCD at any time. *Akt1* mRNA, as measured by quantitative RT-PCR, was reduced ($P < 0.05$) by 23% on d4 of VCD exposure, but returned to control levels on d6 and d8. VCD exposure reduced *Foxo3a* mRNA by 26% on d6 ($P < 0.05$) and by 23% on d8 ($P < 0.1$). These results demonstrate that the earliest observed effect of VCD is an inhibition of phosphorylation and nuclear localization of AKT in the oocyte of primordial and small primary follicles. This event is followed by reductions in KIT and FOXO3 protein subcellular distribution prior to changes in mRNA. Thus, these findings further support that VCD induces ovotoxicity by directly targeting the oocyte through posttranslational inhibition of KIT-mediated signaling components.

follicle, ovary, toxicology

INTRODUCTION

KIT is an oocyte-expressed receptor protein tyrosine kinase [1–3]. The ligand for KIT, KITLG, (also known as Stem Cell factor, Steel Factor) is expressed in granulosa cells [4]. Ovarian KITLG/KIT signaling in primordial and small primary follicles is thought to be essential for oocyte viability and survival in a developmental stage when functional follicle-stimulating hormone receptors are not yet expressed [5, 6]. KITLG stimulation of Postnatal Day (PND) 8 mouse and PND5 rat oocytes demonstrated increased phosphorylation of AKT (downstream of phosphatidylinositol-3 kinase; PIK3), which could be blocked using ACK2, a KIT-neutralizing antibody [7]. Further, treatment of mouse and rat oocytes with the PIK3 inhibitor LY294002 blocked the phosphorylation of AKT induced by KITLG [7]. Thus, binding of KITLG to KIT has been shown to activate the PIK3 signaling pathway. In addition to oocyte viability, members of the PIK3 signaling pathway have been demonstrated to play important roles in primordial to small primary follicle activation and recruitment [6–12].

AKT functions as a central and critical molecule in the PIK3 signaling pathway. *Akt1* mRNA is located in oocytes of primordial and small primary follicles of PND8 and PND12 mouse ovaries, with lower expression in granulosa cells [7]. A serine residue on AKT, Ser^{473}, must be phosphorylated in order for it to be fully activated [13]. It has been shown that Ser^{473} pAKT is highly distributed in the oocyte of rat primordial follicles, with reduced abundance at the primary follicle stage [14]. Once activated, pAKT regulates a host of cellular responses such as cell growth, cell cycle entry, and cell survival.

A key molecule regulated by AKT is a member of the forkhead transcription factor family FOXO3 (also known as FKHR-L1). FOXO3 phosphorylation (pFOXO3) is a downstream event in PIK3 signaling. Phosphorylation of FOXO3 followed KITLG stimulation of PND8 mouse oocytes. Further, this event was prevented by PIK3 inhibition with LY294002 [7]. A role for FOXO3 in regulation of primordial follicle activation and recruitment was demonstrated by Castrillon et al. [9]. Female *Foxo3*-null mice showed an age-dependent decline in reproductive fitness and were sterile by 15 wk of age. This presumably resulted from unregulated recruitment of follicles from the primordial pool. In contrast, oocyte-specific overexpression of *Foxo3* resulted in infertile females as a result of retarded primordial follicle recruitment [10]. Thus, it appears that FOXO3 plays a role in determining the rate of primordial follicle activation/recruitment.

Because the mammalian ovary at birth contains a finite number of primordial follicles that cannot be regenerated [15], depletion of this follicle pool can lead to premature ovarian failure. 4-vinylcyclohexene (VCH) is an occupational chemical formed by dimerization of 1,3-butadiene and is a by-product of the pesticide, rubber, plastic, and flame retardant industries [16]. A metabolite of VCH, 4-vinylcyclohexene diepoxide

[1]Supported by National Institutes of Health grant ES09246, Center Grant 06694, and K99ES016818 from the National Institute of Environmental Health Sciences (to A.F.K.). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Environmental Health Sciences or the National Institutes of Health.
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Received: 5 August 2010.
First decision: 7 September 2010.
Accepted: 5 November 2010.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

(VCD) is used as an industrial diluent for epoxides [17]. VCD is ovotoxic and has been shown to selectively destroy small preantral (primordial and primary) [18–21] follicles in the ovaries of mice and rats via acceleration of atresia (apoptosis) [18, 19, 22–24].

A time course of in vitro VCD (30 μM) exposure of neonatal rat ovaries (highly enriched in primordial and small primary follicles, targeted by VCD) has identified that follicle loss is first seen on Day 6 of culture (d6) [25]. Oligoarray analysis demonstrated that, following follicle loss, mRNA encoding *Kit* was reduced in rat ovaries in response to VCD exposure via in vivo dosing (d15) or in vitro culture (d8) [26].

A study to evaluate a role for PIK3 signaling (downstream of KIT) in VCD-induced ovotoxicity used the PIK3 inhibitor, LY294002 [25]. Inhibition of PIK3 signaling protected the primordial follicle pool from VCD-induced follicle loss, but did not prevent VCD-induced depletion of small primary follicles. Therefore, it was hypothesized that VCD increases recruitment of primordial follicles into the small primary pool as a mode of ovotoxicity [25].

Due to the hypothesized role of the KIT signaling pathway in VCD-induced ovotoxicity, it is of interest to characterize cellular events initiated by VCD. Therefore, this study was designed to investigate the earliest effects of VCD on localization of KIT protein and its downstream PIK3 signaling pathway members—AKT1 and FOXO3—in PND4 F344 rat ovaries at time points prior to VCD-induced follicle loss.

MATERIALS AND METHODS

Reagents

VCD (mixture of isomers, >99% purity), 2-β-mercaptoethanol, 30% acrylamide/0.8% bis-acrylamide, ammonium persulfate, glycerol, N′,N′,N′,N′-tetramethyl-ethylenediamine (TEMED), Tris base, TrisHCL, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (vitamin C), and transferrin were purchased from Sigma-Aldrich Inc. (St Louis, MO). 2-(4-Morpholinyl)-8-phenyl-4*H*-1-benzopyran-4-one (LY294002; CAS# 154447-36-6) was purchased from A.G. Scientific, Inc. (San Diego, CA). Dulbecco modified Eagle medium/nutrient mixture F-12 (Ham) 1× (DMEM/Ham F12), Albumax, penicillin/streptomycin (5000 U/ml and 5000 μg/ml, respectively), Hanks balanced salt solution (without $CaCl_2$, $MgCl_2$, or $MgSO_4$), custom-designed primers, and Superscript III One-Step RT-PCR System were obtained from Invitrogen Co. (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). RNeasy Mini kit, QIAshredder kit, RNeasy MinElute kit, and Quantitect SYBR Green PCR kit were purchased from Qiagen Inc. (Valencia, CA). Anti-KIT antibody was purchased from DAKO (Carpinteria, CA). Anti-AKT and anti-pAKT antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FOXO3 antibody was purchased from Upstate Biotechnology (Lake Placid, NY), and anti-pFOXO3 antibody was purchased from Cell Signaling (Danvers, MA). Goat anti-rabbit secondary antibody was purchased from Pierce Biotechnology (Rockford, IL). Cy-5-streptavidin was obtained from Vector (Burlingame, CA). YOYO-1 was purchased from Molecular Probes (Eugene, OR).

Animals

A breeding colony was established from Fischer 344 (F344) rats that were 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). The animals were provided a standard diet with ad libidum 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.

In Vitro Ovarian Culture

Ovaries from PND4 F344 rats were cultured as previously described [27]. Briefly, PND4 female F344 rats were euthanized by CO_2 inhalation followed by decapitation. Each ovary was removed, trimmed of oviduct and excess tissue, and 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 Albumax, 50 μg/ml ascorbic acid, 5 U/ml penicillin per 5 μg/ml streptomycin, and 27.5 μg/ml transferrin per well in a 48-well plate previously equilibrated to 37°C. Using fine forceps, a drop of medium was placed to cover the top of the ovary 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, media were removed and fresh media and treatment were replaced every 2 days. Ovaries in culture maintained viability throughout the entire time course (previously up to 15 days [27]).

RNA Isolation

Following 2, 4, 6, or 8 days of in vitro culture, ovaries treated with vehicle control or VCD (30 μM) were stored in RNA*later* at −80°C. Total RNA was isolated (n = 3; 10 ovaries per pool) using an RNeasy Mini kit. Briefly, ovaries were lysed and homogenized using a motor pestle followed by applying the mixture onto a QIAshredder column. The QIAshredder column containing ovarian tissue sample was centrifuged at 14 000 rpm for 2 min. The resulting eluant was applied to an RNeasy mini column, allowing RNA to bind to the filter cartridge. Following washing, RNA was eluted from the filter and concentrated using an RNeasy MinElute kit. Briefly, isolated RNA was applied to an RNeasy MinElute spin column, and after washing, RNA was eluted using 14 μl of RNase-free water. RNA concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280nm; NanoDrop technologies, Inc., Wilmington, DE).

First-Strand cDNA Synthesis and Real-Time Polymerase Chain Reaction

Total RNA (0.5 μg) was reverse transcribed into cDNA using the Superscript III One-Step RT-PCR System. Complementary DNA was diluted (1:25) in RNase-free water. Two microliters of diluted cDNA were amplified on a Rotor-Gene 3000 using Quantitect SYBR Green PCR kit and custom-designed primers (*Akt1* forward: 5′-ACCTCTGAGACCGACACCAG-3′, *Akt1* reverse: 5′-AGGAGAACTGGGGAAAGTGC-3′; *Foxo3* forward: 5′-CAAC CAAGGAAATGCTCCTC-3′, *Foxo3* reverse: 5′-CTGTGGCCCTTATCCTT GAA-3′; *Actb* forward: 5′-TCTATCCTGGCCTCACTGTC-3′, *Actb* reverse: 5′-ACGCAGCTCAGTAACAGTCC-3′). The cycling program consisted of a 15-min hold at 95°C and 45 cycles of denaturing at 95°C for 15 sec, 45 cycles of annealing at 58°C for 15 sec, and 45 cycles of extension at 72°C for 20 sec, at which point data were acquired. Product melt conditions were determined using a temperature gradient from 72°C to 99°C, with a 1°C increase at each step. There was no difference in β-actin (ACTB) mRNA between vehicle control and VCD-treated ovaries. Therefore, each sample was normalized to ACTB before quantification. For graphical purposes, only one control value is presented; however, control reactions were performed at each time point, and VCD-induced effects were expressed as a fold change relative to the control on that day.

Protein Isolation

Pools of whole ovarian protein (10 ovaries per pool) homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer as previously described [28]. 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 aliquoted and stored at −80°C until further use. Protein was quantified using a standard bicinchoninic acid protocol on a 96-well assay plate. Emission absorbance values were detected with a λ = 540 nm excitation on a Synergy HT Multi-Detection 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 (10%) was used to separate homogenate proteins (10 μg; n = 3), and these were subsequently transferred onto nitrocellulose membranes as previously described [28]. Briefly, membranes were blocked for 1 h with shaking at 4°C in 5% milk in Tris-buffered saline (TBS) with Tween-20 (TTBS). Membranes were incubated with primary antibody in 5% milk in TTBS overnight at 4°C. Antibody dilutions were anti-KIT (1:400), anti-AKT (1:50), anti-pAKT (1:200), anti-FOXO3 (1:500), anti-pFOXO3 (1:50), anti-Actin (1:1000), and anti-PPIB (peptidylprolyl isomerase B [PPIB]/cyclophilin; 1:2000). 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 TBS. Western blots

were detected by chemiluminescence (using electrochemiluminescence plus chemiluminescence detection substrate) and exposed to x-ray film. Densitometry of the appropriate bands was perfomed using Image J software (http://rsbweb.nih.gov/ij/). Individual treatment values were normalized to one of two loading controls, PPIB or ACTB.

Immunofluorescence Staining and Confocal Microscopy

Following in vitro culture for 2 or 4 days, ovaries treated with vehicle control or VCD (30 μM) were fixed in 4% buffered formalin for 2 h, transferred to 70% ethanol, embedded in paraffin, and serially sectioned, and every 10th section was mounted. Sections were deparaffinized (approximately 10 sections per ovary) and incubated with primary antibodies directed against KIT (1:400 dilution), pAKT (1:50 dilution), FOXO3 (1:100 dilution), or pFOXO3 (1:50 dilution) at 4°C overnight. Specificity for these antibodies was determined after observing a single protein band at the expected molecular weight by Western blotting (data not shown). Blocking solution for immunofluorescence was 5% BSA. Secondary biotinylated antibody was applied for 1 h, followed by CY-5-streptavidin (1 h; 1:100 dilution). Sections were treated with RNase A (100 μg/ml) for 1 h, followed by staining with YOYO-1 (10 min; 5 nM). Slides were repeatedly rinsed with PBS, cover-slipped, and stored in the dark (4°C) until visualization. Primary antibody was not added to immunonegative ovarian sections. Immunofluorescence was visualized on a Zeiss (LSM 510 NLO-Meta) confocal microscope with an argon and helium-neon laser projected through the tissue into a photomultiplier at $\lambda = 488$ and 633 nm for YOYO-1 (green) and CY-5 (red), respectively. All images were captured using a 40× objective lens. Multiple readings were taken throughout the sections. Protein staining was quantified using ImageJ software. Briefly, integrated density of protein staining was measured in five primordial and five small primary follicles per section. Average number of follicles analyzed per ovary was primordial, 139, and small primary, 92.

Statistical Analysis

Comparisons were made between treatments using StatView 5 software (SAS Institute Inc., Cary, NC) ANOVA and Fisher protected least-significant difference multiple range test. Statistical analysis comparing protein immunofluorescence staining in control or VCD-treated ovaries was carried out by comparing integrated density measurements within each staining day (one ovary per staining; n = 3). Statistical analysis was carried out on raw data for each n, and for graphical purposes values were expressed as a percentage of the control treatments (n = 3). The assigned level of significance for all tests was $P < 0.05$, with $P < 0.1$ considered as a trend for a difference.

RESULTS

Effect of VCD Treatment on Akt1 *and* Foxo3 *mRNA over a Time Course of Exposure in PND4 F344 Rat Ovaries*

Previous studies in cultured PND4 F344 rat ovaries determined that follicle loss in response to VCD (30 μM) first occurs on d6 [25]. It was previously reported that, under those same conditions, mRNA encoding *Kit* was first reduced by VCD on d4 of culture [26]. Thus, to further investigate a temporal association with onset of follicle loss, PND4 F344 rat ovaries were cultured with or without VCD (30 μM) for 2–8 days to measure changes in mRNA encoding *Akt1* or *Foxo3*. Relative to control-treated ovaries, *Akt1* mRNA was reduced ($P < 0.05$) on d4 of VCD treatment; however, there was no effect on d6 or d8 (Fig. 1A). *Foxo3* mRNA was unchanged on d2 and d4 of VCD exposure, but was reduced on d6 ($P < 0.05$; Fig. 1B) and d8 ($P < 0.1$; Fig. 1B).

Effect of VCD Exposure on KIT, AKT, pAKT, FOXO3, and pFOXO3 Proteins

The effect of VCD exposure on ovarian KIT, AKT, pAKT, FOXO3, and pFOXO3 protein levels was evaluated by Western blotting or immunoflourescence staining as analyzed by confocal microscopy. The time points chosen for immunofluorescence staining were d2 and d4 of VCD exposure, i.e., times preceding VCD-induced follicle loss (d6). PND4 F344 rat ovaries were cultured in control media with or without VCD (30 μM) for 2–6 days. Relative to control, total ovarian KIT protein was reduced ($P < 0.05$) on d4 but not on d2 of culture (Fig. 2, A and B). Previously, it was shown by immunoflourescence staining that KIT protein is localized to the pericytoplasmic region of the oocyte in PND4 ovaries cultured for 8 days in control medium [26]. On d2 and d4 of culture, KIT protein is also localized to the oocyte pericytoplasmic region in both primordial and small primary follicles (Fig. 2, C–G). As with Western blot analysis, there was no effect of VCD on KIT protein staining on d2 in either primordial or small primary follicles. However, by d4 of VCD exposure, oocyte pericytoplasmic KIT protein staining was reduced ($P < 0.05$) by 44% and 40% in primordial and small primary follicles, respectively (Fig. 2H).

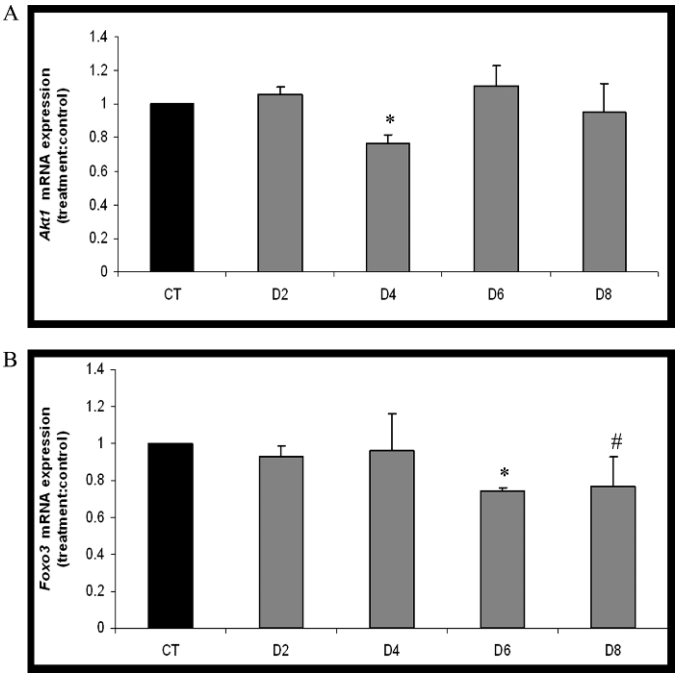

FIG. 1. Temporal effect of VCD exposure on *Akt1* and *Foxo3* mRNA expression. Ovaries from PND4 F344 rats were cultured in vehicle control media (CT) with or without 30 μM VCD for 2–8 days. Following incubation, total RNA was isolated, reverse transcribed into cDNA, and analyzed for *Akt1* (**A**), *Foxo3* (**B**), or *Actb* mRNA expression by RT-PCR as described in methods. Values are mean fold level, relative to a day-matched control ± SEM; n = 3 (8–10 ovaries per pool). $^{*}P < 0.05$; $^{\#}P < 0.1$; different from control.

Total AKT staining was widely distributed throughout the ovary (Fig. 3, A, B, E, and F). In contrast, pAKT staining is primarily localized to the oocyte nucleus in primordial and small primary follicles in cultured PND4 F344 rat ovaries (Fig. 3, C, D, G, and H). There was no effect of VCD on total AKT protein (quantification not shown). Conversely, pAKT oocyte nuclear staining was reduced ($P < 0.05$) on d2 (primordial, 39% below control; small primary follicles, 37% below control; Fig. 3K) and d4 ($P < 0.05$, primordial, 27% below control; $P < 0.1$, 29% small primary follicles, below control; Fig. 3K) of VCD exposure. There was no effect of VCD on pAKT staining in granulosa cells on d2 of exposure (Fig. 3L).

There was no effect of VCD on pAKT protein levels from whole ovarian lysate as measured by Western blotting on d2–d6 (Fig. 4, A and C). Additionally, no effect of VCD on total FOXO3 protein was detected by Western blotting on d2–d6 of VCD exposure (Fig. 4, B and C).

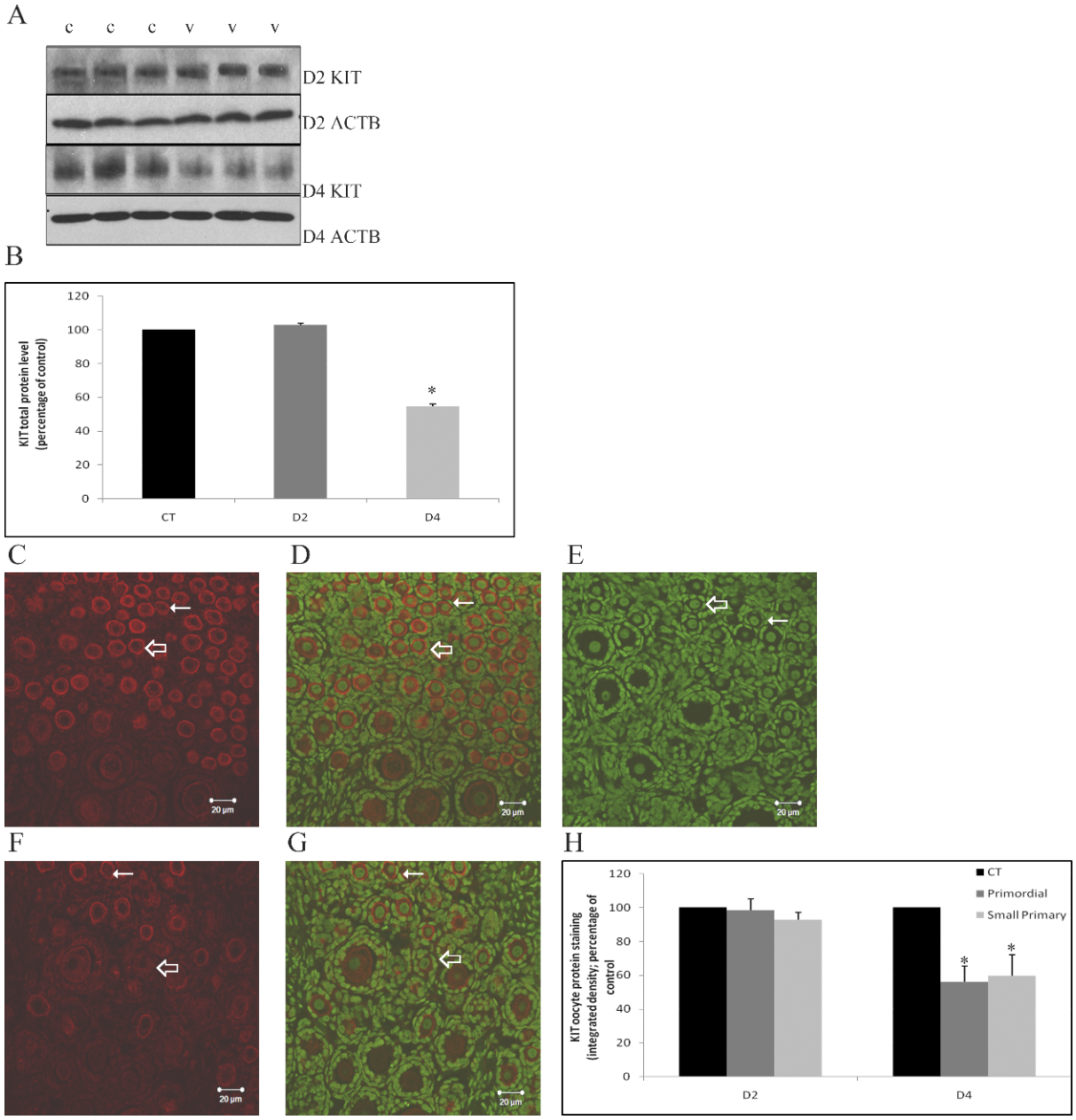

FIG. 2. Effect of VCD on KIT protein staining. PND4 F344 rat ovaries were cultured with vehicle control or 30 μM VCD for 2 or 4 days and processed for Western blotting or immunoflourescence staining with confocal microscopy as described in *Materials and Methods*. **A**) Western blotting for KIT or ACTB protein on d2 and d4 of VCD exposure (control = c; VCD = v). **B**) Quantification of KIT protein staining with normalization to ACTB. Representative images for immunofluorescence staining of KIT protein on d4 are shown. Cy-5 red staining (anti-KIT antibody) for (**C**) control-treated ovary and (**F**) VCD-treated ovary and genomic DNA (green YOYO1 stain) overlay for (**D**) control-treated ovary, (**E**) immunonegative control, and (**G**) VCD-treated ovary at 40× magnification. Thin arrows indicate primordial follicles; thick arrows indicate small primary follicles. Bar = 20 μm. **H**) Quantification of oocyte pericytoplasmic KIT protein staining on d2 and d4 of VCD exposure. Values are percentage of control; n = 3; $^*P < 0.05$. By either method, oocyte plasma membrane staining was reduced by VCD on d4 of incubation, relative to control.

The competitive inhibitor of PIK3, LY294002, was used to confirm that AKT is a downstream phosphorylation target of PIK3 in the oocyte. PND4 F344 ovaries were cultured for 2 or 4 days in control media with or without LY294002 (20 μM). This concentration was previously determined using the in vitro ovary culture method [25]. Immunofluorescence staining demonstrated that, in the presence of PIK3 inhibition, pAKT appeared to be enriched in the cytoplasm of primordial and small primary follicle oocytes, relative to uninhibited PIK3. Additionally, oocyte nuclear pAKT staining intensity was markedly reduced with PIK3 inhibition (Fig. 5).

In primordial and small primary follicles, total FOXO3 protein stained the cytoplasm and nucleus of granulosa cells and oocytes (Fig. 6, A, B, E, and F). Staining was predominantly in the oocyte nucleus. There was no effect of VCD exposure after 2 days on total oocyte FOXO3 protein (cytoplasm and nucleus; Fig. 6K). By d4 of VCD exposure, total FOXO3 protein staining in the oocyte was reduced ($P < 0.05$) by 39% and 36% below control in primordial and small primary follicles, respectively, as compared with control (Fig. 6K). Total FOXO3 protein was not quantified in granulosa cells.

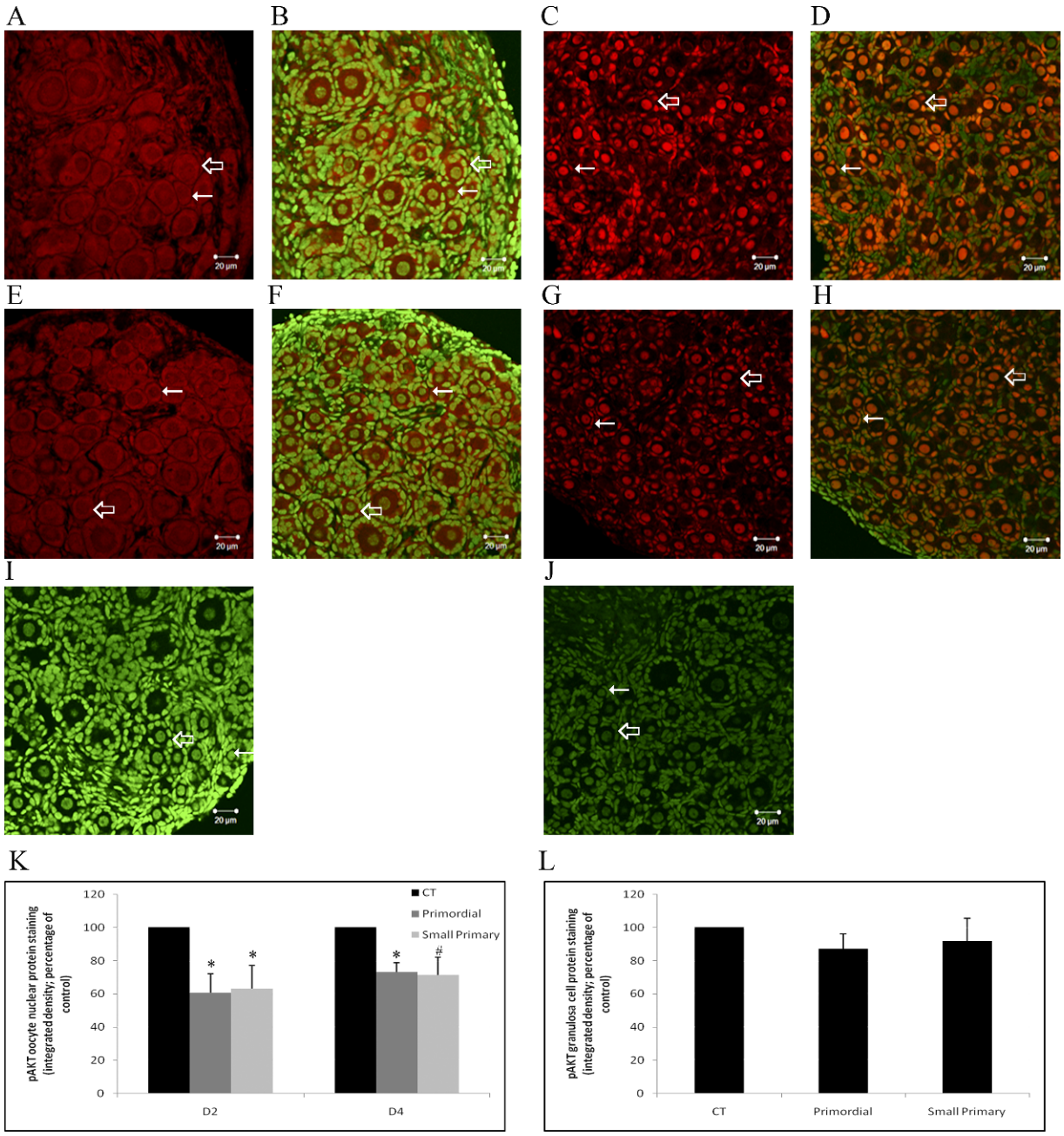

FIG. 3. Effect of VCD on AKT and pAKT protein staining. PND4 F344 rat ovaries were cultured with vehicle control (**A**–**D**, **I**, and **J**) or 30 μM VCD (**E**–**H**) for 2 or 4 days and processed for immunoflourescence staining with confocal microscopy as described in *Materials and Methods*. Representative images for immunofluorescence staining for AKT (**A**, **B**, **E**, **F**, and **I**) or pAKT (**C**, **D**, **G**, **H**, and **J**) on d2 are shown. Cy-5 red staining (anti-AKT antibody) for total AKT protein for (**A**) control-treated ovary and (**E**) VCD-treated ovary and genomic DNA (green YOYO1 stain) overlay for (**B**) control-treated ovary, (**F**) VCD-treated ovary, and (**I**) immunonegative control at 40× magnification. Cy-5 red staining (anti-pAKT antibody) for pAKT protein for (**C**) control-treated ovary and (**G**) VCD-treated ovary and genomic DNA (green YOYO1 stain) overlay for (**D**) control-treated ovary, (**H**) VCD-treated ovary, and (**J**) immunonegative control at 40× magnification. Thin arrows indicate primordial follicles; thick arrows indicate small primary follicles. Bar = 20 μm. Quantification of (**K**) oocyte nuclear pAKT on d2 and d4 and (**L**) granulosa cell pAKT on d2 of VCD exposure. Values are percentage of control; n = 6; $^{*}P < 0.05$; $^{\#}P < 0.1$. Phosphorylated AKT protein levels were reduced on d2 and d4 in the oocyte but not in granulosa cells.

Unlike total FOXO3 protein, phosphorylated FOXO3 (pFOXO3) stained predominantly in the cytoplasm of granulosa cells in large primary and secondary follicles. There was very little pFOXO3 staining in primordial and small primary follicles. Additionally, no protein staining for pFOXO3 was observed in the oocyte of any follicle size (Fig. 6, C, D, G, and H).

DISCUSSION

It was previously determined that mRNA encoding *Kit* is reduced from d4 onwards by VCD in cultured PND4 rat ovaries [26]. The current study demonstrated that VCD also decreases levels of KIT protein on the oocyte pericytoplasmic membrane following d4 of exposure. Whether the d4 decrease in protein resulted from reduced synthesis or from down-

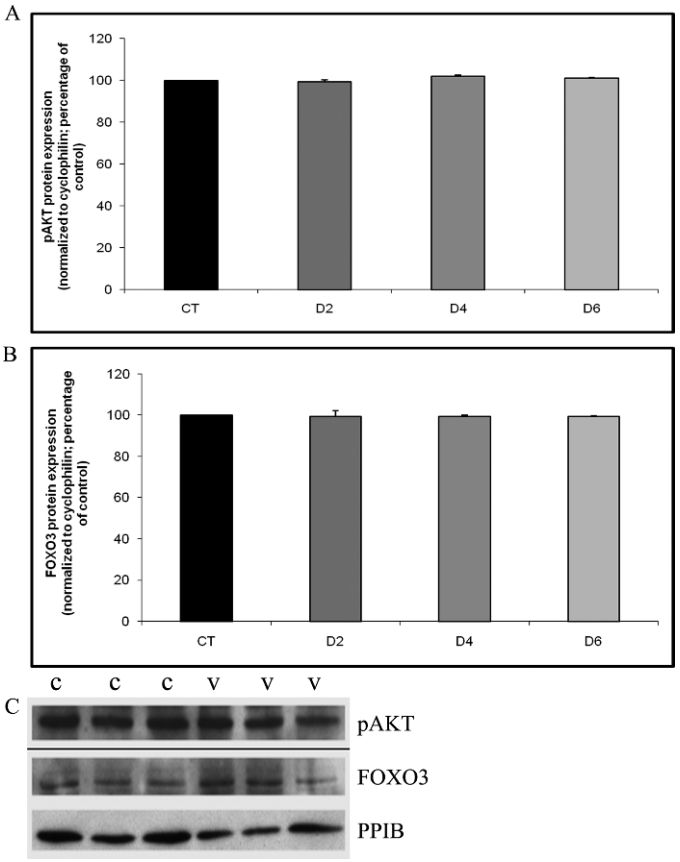

FIG. 4. Effect of VCD exposure on pAKT and FOXO3 protein from whole ovarian lysate. Ovaries from PND4 F344 rats were cultured in vehicle control media (CT) with or without 30 μM VCD for 2–6 days. Following incubation, total protein from whole ovarian lysate was isolated and Western blotting performed to detect pAKT and FOXO3 protein. **A**) Quantification of pAKT protein staining with normalization to PPIB on d2–6 of VCD exposure. **B**) Quantification of FOXO3 protein staining with normalization to PPIB on d2–6 of VCD exposure. **C**) Representative Western blot for pAKT, FOXO3, and PPIB after d2 of culture. Values are percentage of control; n = 3 (control = c; VCD = v). There was no effect of VCD exposure on pAKT or FOXO3 protein level from whole ovarian lysate.

regulation of KIT receptor cannot be determined at this time. However, exogenous KITLG attenuated VCD-induced follicle loss [26], supporting that VCD may interact with membrane-bound KIT to disrupt its signaling pathway.

AKT is activated by phosphorylation downstream of PIK3 [29] and serves as an indirect measurement of PIK3 activity. Genetic depletion of *Akt1* in mice demonstrated a role for AKT in regulating the primordial follicle pool [30]. Relative to wild type, there was a reduction in numbers of growing early antral and antral follicles in $Akt1^{-/-}$ mouse ovaries on PND25. By PND90, a reduction in primordial follicles was also observed. Additionally, oocytes in $Akt1^{-/-}$ primary follicles were larger than those in $Akt1^{+/+}$ mice. This transient follicle phenotype has also been seen in rat neonatal ovaries cultured with exogenous KITLG [26] as well as ovaries from $Foxo3^{-/-}$ mice [9]. These collective observations suggest, therefore, that under these conditions there has been selective activation/recruitment of the oocyte and not of granulosa cells. Furthermore, these findings are in support of a role for the oocyte in dictating primordial follicle activation and recruitment.

In the current study, Western blot analysis showed that VCD caused a decrease in total ovarian KIT protein on d4 of culture. This was consistent with VCD-induced reduction in KIT protein as visualized by immunoflourescence staining. Conversely, no sustained effect of VCD on pAKT protein levels in whole ovarian lysates was observed by Western blot analysis. However, when visualized by immunofluorescent staining there was a reduction in oocyte nuclear pAKT following 2 days of VCD exposure. This event preceded the VCD-induced reduction in KIT protein. Thus, the response of KIT protein to VCD was consistent whether measured by Western blot analysis or immunoflourescence staining. On the other hand, there was a discrepancy between the two methods when measuring pAKT protein. It is likely that because pAKT was observed in granulosa cells as well as oocytes, and because staining in granulosa cells was unaffected by VCD, a decrease in oocyte-specific protein was not detectable by Western blot analysis in whole ovarian protein fractions. This indicates the benefit of using immunoflourescence staining to observe selective ovarian compartmental distribution of various proteins.

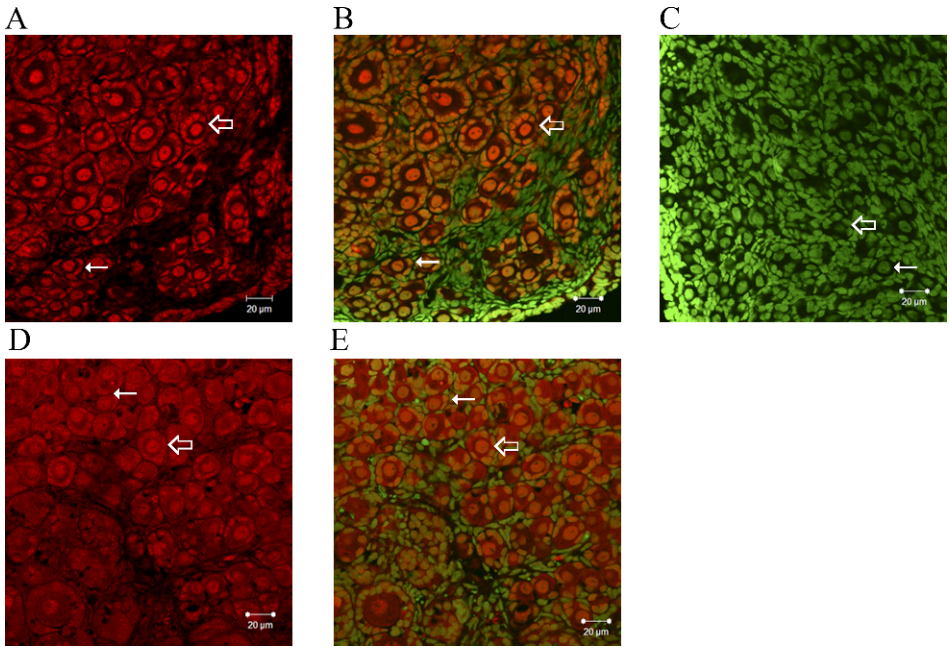

FIG. 5. Effect of PIK3 inhibition on pAKT protein staining. PND4 F344 rat ovaries were cultured with vehicle control (**A**–**C**) or LY294002 (20 μM; **D** and **E**) for 2 days and processed for immunoflourescence staining with confocal microscopy as described in *Materials and Methods*. Representative images show Cy-5 red staining for pAKT protein in (**A**) control-treated ovary and (**D**) LY294002 treated ovary and genomic DNA (green YOYO1 stain) overlay for (**B**) control-treated ovary, (**C**) immunonegative control, and (**E**) LY294002-treated ovary at 40× magnification. Thin arrows indicate primordial follicles; thick arrows indicate small primary follicles. Bar = 20 μm. Overall staining for pAKT became less intense in the cell nucleus and more diffusely distributed in the cytoplasm.

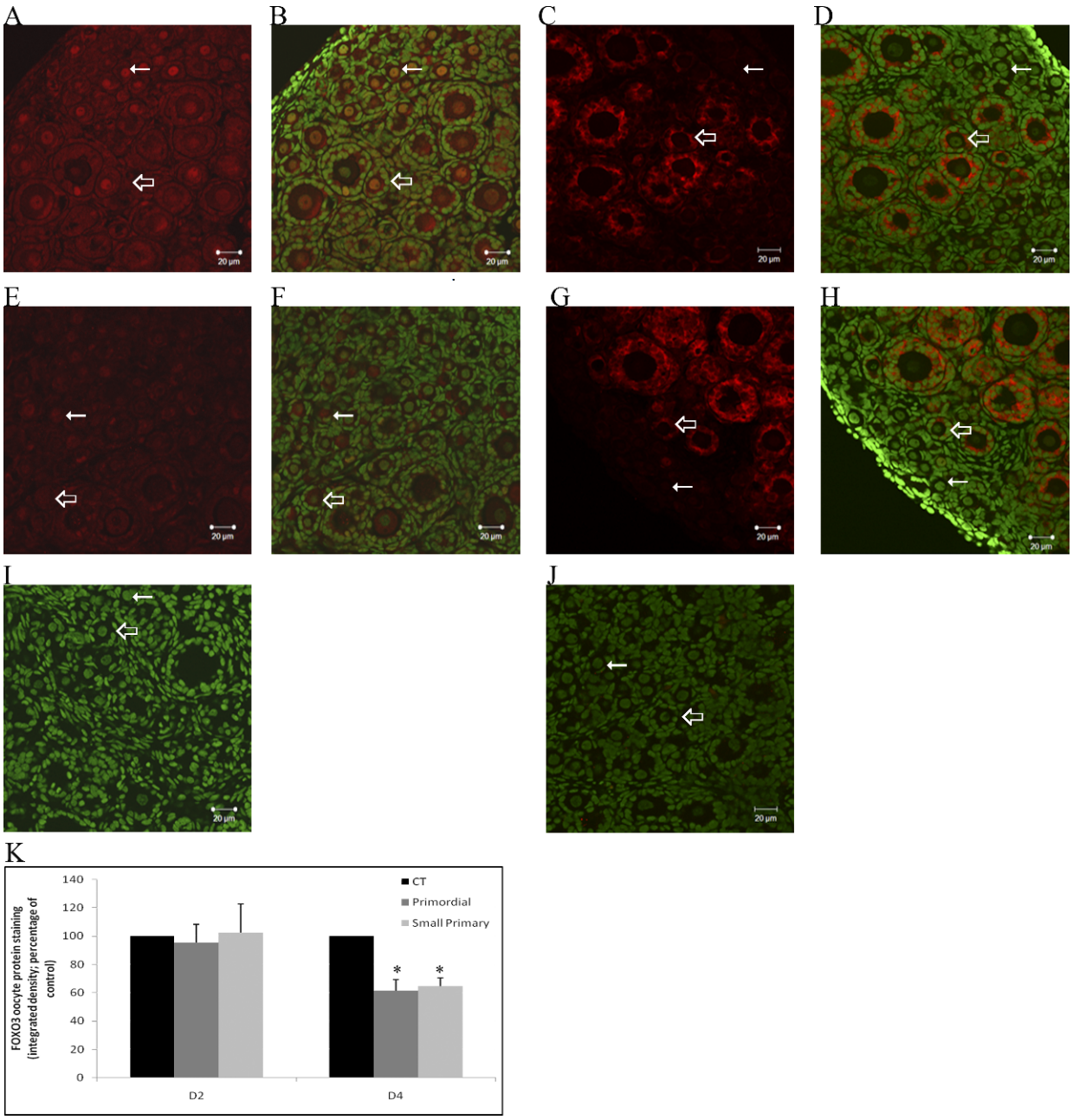

FIG. 6. Effect of VCD on FOXO3 and pFOXO3 protein staining. PND4 F344 rat ovaries were cultured with vehicle control (**A**–**D**) or 30 μM VCD (**E**–**H**) for 2 or 4 days and processed for immunoflourescence staining with confocal microscopy as described in *Materials and Methods*. Representative images for immunofluorescence staining for FOXO3 (**A**, **B**, **E**, **F**, and **I**) and pFOXO3 (**C**, **D**, **G**, **H**, and **J**) on d4 are shown. Cy-5 red staining for total FOXO3 protein for (**A**) control-treated ovary and (**E**) VCD-treated ovary and genomic DNA (green YOYO1 stain) overlay for (**B**) control-treated ovary, (**F**) VCD-treated ovary, and (**I**) immunonegative control at 40× magnification. Cy-5 red staining for pFOXO3 protein for (**C**) control-treated ovary and (**G**) VCD-treated ovary and genomic DNA (green YOYO1 stain) overlay for (**D**) control-treated ovary, (**H**) VCD-treated ovary, and (**J**) immunonegative control at 40× magnification. Thin arrows indicate primordial follicles; thick arrows indicate small primary follicles. Bar = 20 μm. **K**) Quantification of oocyte FOXO3 protein staining on d2 and d4 of VCD exposure. Values are percentage of control; n = 3; $*P < 0.05$. Oocyte FOXO3 protein levels were reduced on d4 of VCD exposure. No pFOXO3 protein expression was detected in primordial or small primary follicle oocytes.

The observed decrease in oocyte nuclear pAKT protein provides evidence of the earliest VCD-induced intracellular event that has been seen to date and may be a downstream response to VCD interaction with the KIT receptor. However, it is possible that VCD interacts with oocyte cytoplasmic proteins that themselves down-regulate PIK3 signaling independent of KIT, and that the decrease in KIT protein is a feedback response. Both of these scenarios are under investigation. In any event, because decreased pAKT (d2) is the earliest observed event caused by VCD, disruption of the KIT-associated signaling pathway by VCD appears to be a key critical event in VCD-induced follicle loss. Interestingly, inhibition of PIK3 did not markedly affect phosphorylation of AKT at Ser^{473}; however, pAKT nuclear translocation was affected, as demonstrated by the increase in cytoplasmic localization of pAKT.

The effect of VCD on a substrate of pAKT, FOXO3, was evaluated. VCD had no effect on *Foxo3* mRNA expression until such time as follicle loss was underway (d6 and d8). Also, there was no effect of VCD on total FOXO3 protein when

measured by Western blotting in whole ovarian lysates. In contrast, immunofluorescence staining revealed a decrease in total FOXO3 protein within oocytes after d4 of VCD exposure. As with pAKT, the inability of Western blot analysis to demonstrate an oocyte-specific response measurable by immunoflourescence staining for total FOXO3 protein is likely due to the fact that overall ovarian distribution of the protein was not affected by VCD.

The reduction in FOXO3 is likely a downstream event that follows the VCD-induced reduction in phosphorylated AKT signaling after 2 days of exposure. As seen in *Foxo3*$^{-/-}$ mice [9], the reduction in oocyte total FOXO3 protein caused by VCD may result from uncoordinated recruitment of primordial follicles into the small primary pool. Previously, it was reported that FOXO3 becomes hyperphosphorylated and undergoes nuclear export as a result of phosphorylation of AKT [11]. In the current study, no staining for pFOXO3 was seen in the oocyte of any follicle type. Instead, pFOXO3 protein staining was observed in the granulosa cells mainly in large primary and secondary follicles, with staining in very few primordial or small primary follicles. Thus, from these studies, it is unlikely that the phosphorylation status of FOXO3 is involved in VCD-induced ovotoxicity.

A number of studies using genetically deficient mice have demonstrated the importance of KIT-initiated PIK3 signaling for primordial follicle activation/recruitment and survival [6–12]. In general, these studies have shown either accelerated primordial follicle activation/recruitment or complete lack thereof, depending upon which gene is affected. In either case, the result is compromised ovarian function. Similar to *Akt*$^{-/-}$ mice, it is possible that reduced pAKT caused by VCD is associated with a compromised primordial follicle pool. Additionally, the finely tuned balancing act that separates primordial follicle viability from activation/recruitment as yet remains poorly understood. If VCD causes an overall reduction in PIK3 signaling, it would be expected that, in the face of active or inhibited PIK3, VCD would have the same overall effect on primordial and small primary follicle number as does VCD alone. However, previous results indicated that when recruitment via PIK3 signaling was inhibited, VCD could not target primordial follicle numbers, supporting a role for increased primordial follicle recruitment during VCD-induced ovotoxicity. Surprisingly, no difference was observed in protein signaling events between primordial and small primary follicles, even though VCD exposure in the face of PIK3 inhibition resulted in different fates for these two follicle types. Thus, whether VCD affects primordial follicle viability, recruitment, or both remains to be determined.

The current study demonstrates that the initial ovotoxic effects of VCD are not associated with changes in mRNA encoding the *Kit*, *Akt1*, or *Foxo3* genes. Rather, the earliest events involve alterations in the subcellular distribution of KIT (oocyte pericytoplasmic membrane), pAKT (oocyte nucleus), and FOXO3 (oocyte nucleus + cytoplasm) proteins. These results, therefore, support a posttranslational protein signaling pathway as the direct target of VCD, with decreases in transcription of these genes as subsequent responses. The changes seen in these signaling pathway members prior to observed VCD-induced follicle loss also indicate that the oocyte, not the granulosa cell, in primordial and primary follicles is the direct target of VCD.

In summary, the collective findings support the hypothesis that VCD interacts directly with membrane-bound KIT receptor or its downstream signaling pathway in the oocyte as an initial event in its mode of action. In addition to an enhanced understanding of chemical-induced ovotoxicity, these results support the use of VCD as an effective tool to further identify mechanisms involved in primordial follicle recruitment.

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