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Ovarian expressed microsomal epoxide hydrolase: role in detoxification of 4-vinylcyclohexene diepoxide and regulation by phosphatidylinositol-3 kinase signaling

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Abstract

4-vinylcyclohexene diepoxide (VCD) is a metabolite of 4-vinylcyclohexene (VCH) which has the potential to be formed in the ovary through CYP2E1 activity. VCD specifically destroys primordial and small primary follicles in the rodent ovary. Mouse ovaries exposed to VCD demonstrate increased mRNA and protein expression of microsomal epoxide hydrolase (mEH), and an inactive tetrol metabolite (4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane) can be formed in mouse ovarian follicles, potentially through detoxification action of mEH. In contrast, mEH can bioactivate another ovotoxic chemical, 7,12-dimethylbenz[a]anthracene (DMBA) to a more toxic compound, DMBA-3,4-diol-1,2-epoxide. Thus, the present study evaluated a functional role for mEH during detoxification of VCD. Additionally, because inhibition of the phosphatidyinositol-3 kinase (PI3K) signaling pathway in a previous study protected primordial follicles from VCD-induced destruction, but accelerated DMBA-induced ovotoxicity, a role for PI3K in ovarian mEH regulation was evaluated. Using a post-natal day (PND) 4 Fischer 344 rat whole ovary culture system inhibition of mEH using cyclohexene oxide during VCD exposure resulted in a greater ($P < 0.05$) loss of primordial and small primary follicles relative to VCD-treated ovaries. Also, relative to controls, *meh* mRNA was increased ($P < 0.05$) on day 4 of VCD (30 μM) exposure, followed by increased ($P < 0.05$) mEH protein after 6 days. Furthermore, inhibition of PI3K signaling increased mEH mRNA and protein expression. Thus, these results support a functional role for mEH in the rat ovary, and demonstrate the involvement of PI3K signaling in regulation of ovarian xenobiotic metabolism by mEH.

Keywords

microsomal epoxide hydrolase; 4-vinylcyclohexene; phosphatidylinositol-3 kinase; ovary; primordial follicle

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Introduction

The ovary is the primary reproductive organ in females, and contains follicles at various stages of development. The primordial follicle is the most immature form, and consists of a meiotically-arrested oocyte surrounded by a single layer of squamous granulosa cells. Females are born with a finite number of primordial follicles, which once depleted, cannot be replaced (Hirshfield, 1991). The phosphatidylinositol-3 kinase (PI3K) signaling pathway is critically involved in maintaining primordial follicle viability (Parrott and Skinner, 1999; Yoshida *et al*., 1997) and regulates the rate at which primordial follicles are recruited into the growing follicular pool (Yoshida *et al*., 1997; Kissel *et al*., 2000; Castrillon *et al*., 2003; Reddy *et al*., 2005; John *et al*., 2008; Reddy *et al*., 2008). This survival pathway is activated when granulosa cell-expressed Kit Ligand (KITL) binds to the oocyte expressed receptor, c-KIT (Ismail *et al*., 1996). In response, c-KIT undergoes autophosphorylation, which leads to activation of PI3K (Castrillon *et al*., 2003; Reddy *et al*., 2005; John *et al*., 2008; Liu *et al*., 2006; Reddy *et al*., 2008), eventually resulting in phosphorylation of a key downstream molecule, AKT (Nicholson and Anderson, 2002; Datta *et al*., 1999).

Exposure to a chemical that accelerates depletion of the finite primordial follicle pool can lead to premature ovarian failure. 4-vinylcyclohexene (VCH) is a byproduct of the pesticide, rubber, plastic and flame retardant industries (Rappaport and Fraser, 1977). Bioactivation of VCH to the ovotoxic form, 4-vinylcyclohexene diepoxide (VCD) occurs via the cytochrome P450 family of enzymes, including ovarian expressed CYP 2E1 (Cannady *et al*., 2002; 2003; Rajapaksa *et al*., 2007a). Human exposure to VCH and VCD are limited, however, VCD is a useful model ovotoxicant due to its capacity to selectively destroy primordial and small primary follicles in ovaries of mice and rats (Smith *et al*., 1990; Doerr *et al*., 1985).

The enzyme microsomal epoxide hydrolase (mEH) is expressed in multiple tissues, including the ovary, and has wide substrate specificity (Dannan and Guengerich, 1982; Mukhtar *et al*., 1978). mEH mRNA and enzyme activity expression were shown to increase in small pre-antral follicles of mouse ovaries following repeated daily dosing (15d) with VCD (0.57 mmol/kg/day) (Cannady *et al*., 2002). Furthermore, mEH mRNA and protein were up-regulated in cultured postnatal day 4 (PND4) $B6C3F_1$ mouse ovaries in response to VCD exposure (Keating *et al*., 2008a), and formation of the inactive tetrol metabolite [4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane] in ovarian follicles of VCD dosed mice has been demonstrated (Flaws *et al*., 1994). Based on these collective data, a functional role for mEH in VCD detoxification is hypothesized but has not yet been established.

In contrast to VCD, the ovotoxic polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA), liberated during combustion of organic matter (Gelboin, 1980), destroys follicles at all stages of development (Mattison and Schulman, 1980). A study in which an ovotoxic index (concentration required for 50% primordial follicle loss) was calculated found that DMBA is approximately 20 times more ovotoxic than VCD (Borman *et al*., 2000). It has been demonstrated that DMBA is bioactivated to a more potent ovotoxicant DMBA-3,4-diol, 1,2-epoxide via the mEH enzyme (Rajapaksa et al., 2007; Igawa et al., 2009). Thus, mEH is involved in bioactivation of DMBA and the action of mEH enhances the ovotoxic effects of DMBA.

Inhibition of PI3K protects primordial but not small primary follicles from VCD-induced destruction (Keating *et al*., 2009). In contrast, ovarian DMBA exposure during inhibition of PI3K accelerates the amount of ovotoxicity observed (Keating *et al*., 2009). Due to the divergent role of mEH in bioactivation of DMBA and potential detoxification of VCD, it is proposed that changes in mEH action may be involved in the different levels of ovotoxicity observed with these two chemicals during PI3K inhibition (Figure 1). The purpose of this

study therefore was to 1) establish that mEH has a functional role in VCD detoxification and, 2) to investigate if the previously observed divergent levels of ovotoxicity induced by VCD and DMBA that occur during PI3K inhibition can be attributed to changes in ovarian mEH expression.

Materials and Methods

Reagents

4-vinylcyclohexene diepoxide (VCD), bovine serum albumin (BSA), ascorbic acid, transferrin, cyclohexene oxide (CHO), 2-β-mercaptoethanol, 30% acrylamide/0.8% bisacrylamide, ammonium persulphate, glycerol, N'N'N'N'-Tetramethylethylenediamine (TEMED), Tris base, Tris HCL, sodium chloride, Tween-20 were purchased from Sigma-Aldrich Inc. (St Louis, MO). Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) 1x (DMEM/Ham's F12), Albumax, penicillin (5000U/ml), Hanks' Balanced Salt Solution (without $CaCl_2$, $MgCl_2$ or $MgSO_4$) from Invitrogen Co. (Carlsbad, CA). Millicell-CM filter inserts and 48 well cell culture plates were obtained from Millipore (Bedford, MA) and Corning Inc. (Corning, NY) respectively. RNeasy Mini kit, QIA shredder kit, RNeasy Min Elute kit, and Quantitect™ SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). RNA*later* was obtained from Ambion Inc. (Austin, TX). With the exception of 18S rRNA primers, all primers were obtained from IDT (Coralville, IA). The 18S rRNA primer was obtained from Applied Biosystems (Carlsbad, CA). 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002; CAS#154447-36-6) was purchased from A.G. Scientific, Inc. (San Diego, CA). The mEH antibody was purchased from Detroit R and D (Detroit, MI). Donkey anti-goat secondary antibody was purchased from Vector (Burlingame, CA). The polyclonal β-actin and goat anti-rabbit secondary were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA) and Pierce Biotechnology (Rockford, IL) respectively. Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK).

Animals

All animals were housed one per cage in plastic cages and maintained in a controlled environment (22 ± 2°C; 12h light/12h dark cycles). The animals were provided a standard diet with *ad libidum* access to food and water, and allowed to give birth. All animal experimental procedures were approved by the University of Arizona and Iowa State University Institutional Animal Care and Use Committees.

In vitro ovarian cultures

Ovaries were collected from PND4 female F344 rats and cultured as described by Devine *et al*., 2002. Briefly, PND4 female F344 rat pups were euthanized by CO_2 inhalation followed by decapitation. Ovaries were removed, trimmed out of oviduct and other excess tissues and placed onto Millicell-CM membrane floating on 250 μl of DMEM/Ham's F12 medium containing 1 mg/ml BSA, 1 mg/ml Albumax, 50 μg/ml ascorbic acid, 5 U/ml penicillin and 27.5 μg/ml transferrin per well in a 48 well plate previously equilibrated to 37°C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were treated with vehicle control medium (1% DMSO), VCD (30 μM), the PI3K inhibitor LY294002 (20 μM), and/or the mEH inhibitor CHO (2 mM) and maintained at 37°C and 5% CO_2 for 2-8 days. The concentrations of LY294002 and CHO were previously determined to be effective in the PND4 rat ovary culture system (Keating *et al*., 2009; Igawa *et al*., 2009, respectively). For treatments lasting for more than two days, media was changed every alternate day. The concentration (30 μM) and times of VCD exposure (day 6) were previously determined to

cause 50% primordial and small primary follicle loss (Devine *et al*., 2002; Keating *et al*., 2009).

Histological evaluation of follicle numbers

Following treatment, ovaries were placed in Bouin's fixative for 1.5 hr, paraffin embedded and serially sectioned (5 μM). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 12th section. Unhealthy follicles were distinguished by their granulosa cell content of pyknotic bodies and intense eosinophilic staining of oocytes (Devine *et al*., 2002). Follicle population classification was performed as previously described (Keating *et al*., 2009).

RNA isolation and polymerase chain reaction (PCR)

Following 2, 4, 6 or 8 days of *in vitro* culture, ovaries were stored in RNA later at −80°C. Total RNA was isolated from ovaries (n=3; 10 ovaries per pool) using an RNeasy Mini kit according to the manufacturer's instructions. RNA was eluted in 14 μl of RNase-free water and concentration quantified using a NanoDrop (λ=260/280 nm; ND 1000; Nanodrop Technologies Inc, Wilmington, DE). Total RNA (500 ng) was reverse transcribed to cDNA using Superscript III One- Step RT-PCR System (Invitrogen). Genes of interest were amplified using an Eppendorf mastercycler (Hauppauge, NY) using a Quantitect™ SYBR Green PCR kit (Qiagen Inc.Valencia, CA). The primers used were: *meh* forward primer: 5′GGC ATC GTC CAT AAA CA; *meh* reverse primer: 5′ TCT TCA AAG GCA GCA AAG TG, (NCBI GenBank accession number M26125), *β-actin* forward primer: 5′ TCT ATC CTG GCC TCA CTG TC; *β-actin* reverse primer: 5′ACG CAG CTC AGT AAC AGT CC, (NCBI GenBank accession number NM_007393) and commercially available primers to detect *18S* rRNA. A melting curve analysis was used to ensure that a single product was amplified for each primer set. For the VCD experiments, there was no effect of VCD on β-actin mRNA expression, thus, *meh* mRNA was normalized to *β-actin*. Inhibition of PI3K did result in decreased *β-actin* mRNA expression, thus, *18S* rRNA was used as a housekeeping gene for the PI3K inhibition experiments, since there was no impact of PI3K inhibition on *18S* rRNA expression. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Pfaffl, 2001; Livak and Schmittgen, 2001). The PCR conditions used were: 15 min hold at 95°C and 40 cycles of: denaturing at 95°C for 15 s, annealing at 58°C for 15 s, and extension at 72°C for 15 s.

Protein isolation and Western blot analysis

Following 4, 6 or 8 days of culture, protein was isolated from ovaries (n=3; 10 ovaries/pool) as previously described (Thompson *et al*., 2005). Protein concentration was measured using a standard BCA protocol. Emission absorbance values were detected with a λ=540 nm excitation on a Synergy™ HT Multi-Detection Microplate Reader using KC4™ software (BioTek^{R} Instruments Inc. Winooski, VT).

SDS-PAGE (10%) was used to separate proteins (10 μg; n=3) in the homogenates followed by transfer onto nitrocellulose membranes as previously described (Thompson *et al*., 2005). Membranes were blocked for 1 h with shaking at 4°C in 5% milk in Tris-buffered saline with Tween-20 (TTBS). Membranes were incubated with primary antibody in 5% milk in TTBS overnight at 4°C. The mEH antibody dilution used was 1:2000. Following three washes (10 min) in TTBS, membranes were incubated in HRP-conjugated secondary antibody (1:2000) for 1hr at room temperature. Membranes were washed three times (10 min) in TTBS followed by a single wash for 10 min in Tris Buffered Saline (TBS). Membranes were incubated in chemiluminescence detection substrate (ECL plus) for 5 min and exposed to X-ray film. Densitometry of the appropriate bands was performed using

ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and mEH protein was normalized to Ponceau S densitometry values.

Statistical analysis

Comparisons were made between treatments for follicle count experiments using Statview software analysis of variance (ANOVA). Quantitative RT-PCR and Western blotting data were analyzed by paired t-tests comparing treatment with control raw data at each individual time-point using Prism 5.04 software (GraphPad Software). Statistical significance was defined as $P < 0.05$. For graphical purposes, protein expression is presented as a percentage of the respective controls, and only one control value of 100% is presented.

Results

Effect of mEH inhibition on VCD-induced ovotoxicity in PND4 rat ovaries

Cyclohexene oxide (CHO) is a competitive inhibitor of mEH. In order to evaluate a functional role for mEH in VCD-induced ovotoxicity, F344 rat ovaries were cultured in vehicle control or VCD (30 μM) ± CHO (2 mM) for 8 days, followed by histological evaluation of follicles. This time-point was chosen since primordial and small follicle destruction is established at this time (Keating *et al*., 2009). There was no effect of CHO on primordial follicle number. Relative to control, VCD depleted ($P < 0.05$) primordial follicles by 74.5%. When ovaries were exposed to VCD in the presence of mEH inhibition (CHO), there was greater ($P < 0.05$) primordial follicle loss (36% fewer follicles) relative to VCD alone (Figure 2). Surprisingly, there was an effect of CHO on small primary follicle number (32.5% fewer follicles than control treatment; $P < 0.05$). As expected, relative to control, VCD depleted ($P < 0.05$) small primary follicles (57.5% fewer follicles). Inhibition of mEH in the presence of VCD resulted in additional loss ($P < 0.05$) of small primary follicles compared to VCD alone (55% fewer small primary follicles; Figure 2).

Effect of VCD exposure on meh mRNA in PND4 rat ovaries

It has previously been determined that VCD exposure (30 μM) causes significant loss of both primordial and small primary follicles from 6 days of exposure onwards in cultured PND4 F344 rat ovaries (Keating *et al*., 2009). To determine the temporal pattern of *meh* mRNA expression in response to VCD exposure, PND4 F344 ovaries were cultured in VCD (30 μM) for 2-8 days (Figure 3). Relative to control treated ovaries, there was no change in *meh* mRNA level following 2 days of VCD exposure. On day 4 of VCD exposure, there was an increase ($P < 0.05$) in *meh* mRNA expression (0.61-fold increase) and a trend for increased *meh* mRNA after 6 days (0.51-fold, $P = 0.08$; Figure 3).

Effect of VCD exposure on mEH protein in PND4 rat ovaries

Since mEH mRNA level did not change until 4 days after VCD exposure, changes in mEH protein levels were investigated on days 4, 6 and 8 (Figure 4). There was no effect of VCD on mEH protein level at the 4 day time-point, but mEH protein was increased ($P < 0.05$) after 6 and 8 days of VCD exposure, by 26 and 17%, respectively, relative to control.

Effect of PI3K inhibition on ovarian expression of mRNA encoding meh

To investigate a potential role of PI3K signaling in regulation of *meh*, PND4 F344 ovaries were cultured in vehicle control ± LY294002 (20 μM; PI3K inhibitor) for 2 or 4 days followed by quantitative RT-PCR to determine changes in mRNA encoding *meh*. The housekeeping gene *18S* rRNA was used as an internal control because PI3K inhibition affected the mRNA level for *β-actin* (data not shown). There was a decrease ($P < 0.05$) in *meh* mRNA expression following 2 days of PI3K inhibition (0.51-fold decrease) compared

to vehicle control. In contrast, *meh* mRNA expression was increased ($P < 0.05$) by PI3K inhibition after 4 days (1.21-fold increase; Figure 5).

Effect of PI3K inhibition on ovarian expression of mEH protein

To evaluate the effect of PI3K inhibition on mEH protein, PND4 F344 rat ovaries were cultured in vehicle control ± LY294002 for 4 and 6 days (time-points lagging the mRNA increase) followed by Western blotting to detect mEH protein levels. Due to the observed effects of PI3K on the mRNA encoding the cytoskeleton protein, β-actin, Ponceau S staining was used to confirm equal protein loading across samples and densitometry values for mEH were normalized to those of Ponceau S. mEH protein was significantly increased ($P < 0.05$) by PI3K inhibition by 221% on day 4 and 14% on day 6 relative to control-treated ovaries (Figure 6).

Discussion

Previously, it has been shown that VCD exposure to cultured PND4 B6C3F$_1$ mice resulted in increased mEH mRNA and protein levels (Keating *et al*., 2008a). It has also been demonstrated that follicles isolated from ovaries of VCD-dosed mice produce an inactive tetrol metabolite, [4-(1,2-dihydroxy)ethyl-1,2-dihydroxycyclohexane] (Flaws *et al*., 1994), potentially through the action of mEH. Demonstration of a functional role for mEH during VCD detoxification, however, has been lacking, therefore this study investigated a functional effect of mEH during VCD exposure, using the competitive inhibitor of mEH, cyclohexene oxide (CHO), followed by assessment of follicle number. CHO has previously been determined to be effective in the ovary culture system at the concentration used (Igawa *et al*., 2009). As expected, VCD depleted primordial and small primary follicle number. While there was no impact on primordial follicle number, surprisingly, small primary follicle number was reduced by mEH inhibition (in the absence of VCD). mEH protein is distributed throughout the rat ovary, including the cytoplasm of oocytes, and in granulosa and interstitial cells (Igawa *et al*., 2009). In humans, mEH protein has been detected in granulosa and theca interna cells, where inhibition of mEH in cultured granulosa cells was found to result in decreased Estradiol (E_2) production from testosterone (Hattori *et al*., 2000), independent of an effect on aromatase activity. Female aromatase knockout mice (ArKO) are deficient in E_2 and have higher numbers of primary follicles relative to age matched control mice indicating increased activation from the primordial follicle pool (Britt *et al*., 2002). Thus, E_2 may inhibit primordial follicle activation supporting that the decreased number of small primary follicles due to mEH inhibition in the current study may reflect some increased recruitment from the small primary pool as a consequence of decreased estradiol production. When mEH was inhibited in the presence of VCD, relative to VCD alone, there was greater loss of both primordial follicles and small primary follicles, and loss of small primary follicles was greater than that caused by CHO alone. Since inhibition of mEH would result in exposure to greater persistent concentrations of VCD, these data provide confirmation that ovarian mEH is involved in detoxification of VCD.

meh mRNA was increased in cultured rat ovaries in response to VCD, following 4 days of exposure. Additionally, an increase in mEH protein was observed to follow the increase in mRNA (d4 mRNA; d6 protein). Interestingly, the rise in *meh* mRNA expression was not sustained, despite continuous exposure to VCD. However, mEH protein levels remained elevated, indicating either an increase in mEH protein expression or an extension of mEH protein half-life. Thus, these data confirm that mEH expression is activated in PND4 cultured rat ovaries in a temporal response to VCD exposure. It is likely that despite the increase in ovarian mEH level, that continuous exposure (every two days) to VCD overwhelms the capacity of mEH to detoxify VCD, leading to loss of primordial and small primary follicles,

In a previous study, inhibition of PI3K signaling resulted in decreased ovotoxicity induced by VCD, but accelerated DMBA-induced ovotoxicity (Keating *et al*., 2009). These results suggested that an alteration in metabolism of VCD and DMBA might be occurring. Further, a role for mEH in mediating the divergent ovotoxic effects of VCD and DMBA in CYP 2E1 null mice has been reported (Keating *et al*., 2008b). Thus, mEH is an ovarian expressed enzyme whose action may determine the extent of ovotoxicity caused by exposure to different xenobiotic chemicals.

To further investigate regulation of mEH expression in ovarian tissue, a specific inhibitor of PI3K signaling, LY294002, was employed. After two days in culture, *meh* mRNA was decreased, potentially due to a lag in transcriptional activation. Conversely, PI3K inhibition resulted in increased *meh* mRNA on days 4. mEH protein was increased by PI3K inhibition on days 4 and 6. These results indicate that mEH is downstream of PI3K signaling. Whether PI3K signaling is altered during mEH inhibition by CHO is unclear at this point, but is possible considering the effect of mEH deficiency on small primary follicle number. Increased mEH mRNA and protein supports that increased VCD detoxification and DMBA bioactivation are likely to be involved in the previously observed reduction in VCD-induced follicle loss and the increase in DMBA-induced follicle loss observed with PI3K inhibition (Keating *et al*., 2009).

The PI3K pathway has been identified as a major initial target of VCD (Keating *et al*., 2011). VCD exposure to cultured PND4 rat ovaries reduces c-KIT phosphorylation (Mark-Kappeler *et al*., 2011), resulting in downstream post-translational reductions in oocyte phosphorylated AKT (pAKT) and FOXO3 proteins (Keating *et al*., 2011). Depressed PI3K signaling by VCD exposure is consistent with the increase in mEH protein caused by both VCD and PI3K inhibition reported in the current study. Insulin has been reported to positively regulate hepatic mEH expression (Thomas *et al*., 1989 Kim *et al*., 2003; Kim and Novak, 2007) while glucagon inhibits mEH expression (Kim *et al*., 2003). Induction of Type I diabetes, characterized by insulin deficiency, in rats resulted in approximately 71% less mEH activity compared to the littermate controls. Treatment with insulin restored the activity of mEH. In addition, starvation, a physiological state known to reduce insulin levels, reduced mEH activity by approximately 33% of the control values in rats, while re-feeding restored mEH to control levels (Thomas *et al*., 1989). Thus, insulin plays a role in induction of mEH activity. It has also been shown that treatment of cultured primary rat hepatocytes with insulin increased mEH mRNA and protein in a time- and concentration-dependent manner (Kim *et al*., 2003), while use of the PI3K inhibitors, Wortmannin and LY294002, reduced pAKT level and prevented the insulin-induced increase in mEH (Kim et al., 2003), supporting that PI3K signaling is involved in insulin-induced hepatic regulation of mEH. Use of mTOR inhibitor, rapamycin, also prevented the insulin-induced induction of mEH protein (Kim et al., 2003). Recently, mTOR phosphorylated at position Ser^{2448} (activated form) was demonstrated to be increased by DMBA exposure in neonatal mouse ovaries (Sobinoff *et al*., 2011). Another study has demonstrated the involvement of PI3K signaling in induction of mEH through the transcription factors C/EBPα and C/EBPβ (Ki and Kim, 2008). Taken together, these data support a role for PI3K signaling in regulation of ovarian metabolism by mEH, potentially through the action of mTOR or C/EBP transcription factors, and also support that mEH is a downstream target of PI3K. Whether over-stimulation of PI3K signaling would further alter ovarian metabolism of VCD and DMBA is unclear at this point, however there are indications that altered insulin signaling (through PI3K) as seen during diabetes, can impact mEH expression in extra-ovarian tissues (Thomas *et al*., 1989; Kim and Novak, 2007).

In summary, a functional role for ovarian expressed mEH in VCD detoxification has been demonstrated, along with the temporal response of mEH mRNA and protein to VCD

exposure. Results from the current study also support that PI3K signaling impacts mEH expression. These results further underline the potential impact of ovarian xenobiotic metabolism on the extent of follicular damage during chemical-induced ovotoxicity.

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Highlights of this research

- Ovarian mEH functions to metabolize VCD to a less toxic compound
- mEH expression is increased in a temporal pattern in response to VCD exposure
- PI3K signaling is involved in regulation of ovarian mEH expression

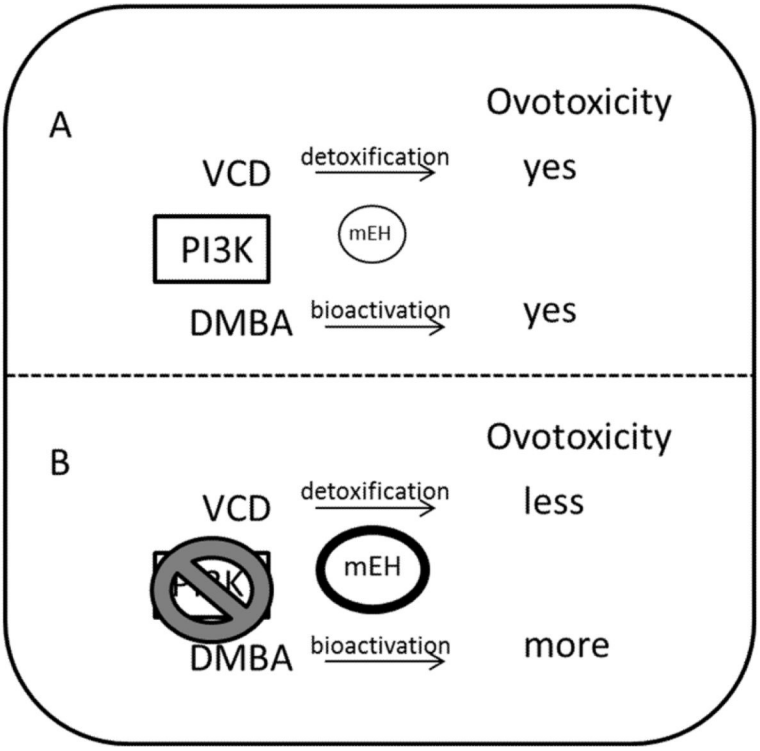

Figure 1. Proposed effect of PI3K inhibition on VCD- and DMBA-induced ovotoxicity

PI3K signaling is proposed to regulate mEH expression. (A) VCD and DMBA both cause depletion of primordial follicles, however, (B) inhibition of PI3K signaling repressed VCD-induced but accelerated DMBA-induced ovotoxicity (Keating *et al*., 2009). These events are proposed to be due to increased mEH expression (B) since mEH is proposed to detoxify VCD, and is known to bioactivate DMBA (Igawa *et al*., 2009). Thus, the divergent levels of ovotoxicity observed due to inhibited PI3K signaling during VCD and DMBA exposure are hypothesized to be due to increased detoxification of VCD, but increased bioactivation of DMBA.

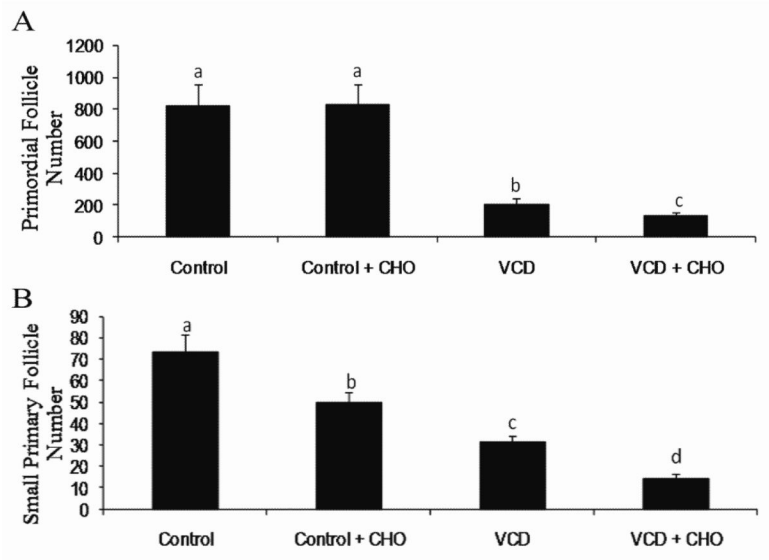

Figure 2. Effect of mEH inhibition on VCD-induced follicle loss

PND4 F344 rat ovaries were cultured in media containing vehicle control or VCD (30 μM), ± CHO (2 mM) for 8 days. Ovaries were processed for histological evaluation and healthy follicles were classified and counted as described in methods. Values are expressed as mean ± SE total follicles counted/ ovary, n=5. Different letters indicate significant difference; $P < 0.05$.

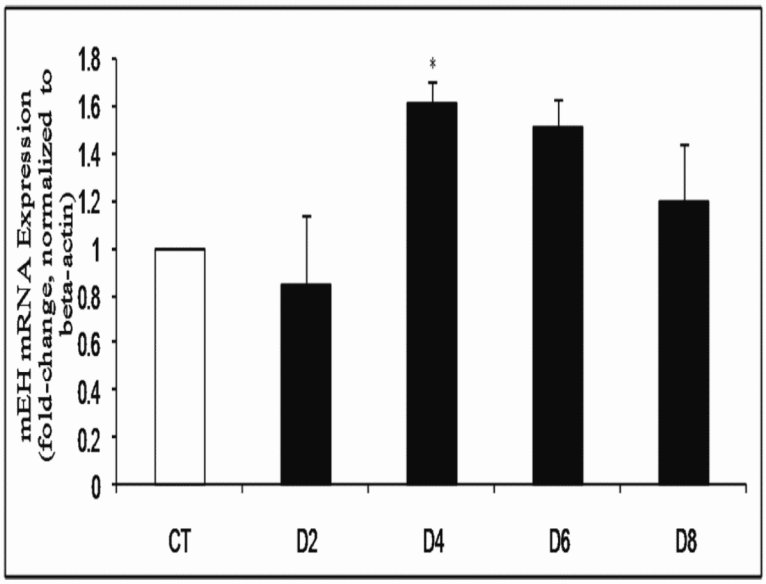

Figure 3. Temporal effect of VCD on *meh* mRNA expression

PND4 F344 rat ovaries were cultured in media containing vehicle control (CT) or VCD (30 μM) for 2-8 days. Following incubation, total RNA was isolated and *meh* and *β-actin* mRNA levels were quantified by RT-PCR as described in methods. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). * $P < 0.05$; different from control.

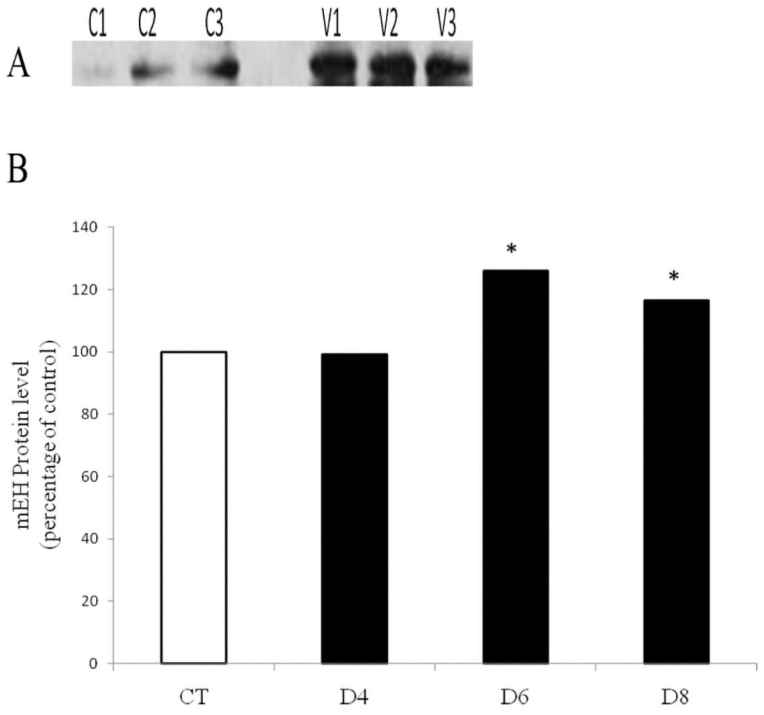

Figure 4. Temporal effect of VCD on mEH protein expression

PND4 F344 rat ovaries were cultured in media containing vehicle control (CT) or VCD (30 μM) for 4-8 days. Total protein was isolated and Western blotting was performed for mEH protein as described in methods. (A) Representative Western blot day 6; Control = C; VCD = V. (B) Values are expressed as a percentage of control mean ± SE; n=3 (10 ovaries per pool). * $P < 0.05$; different from control.

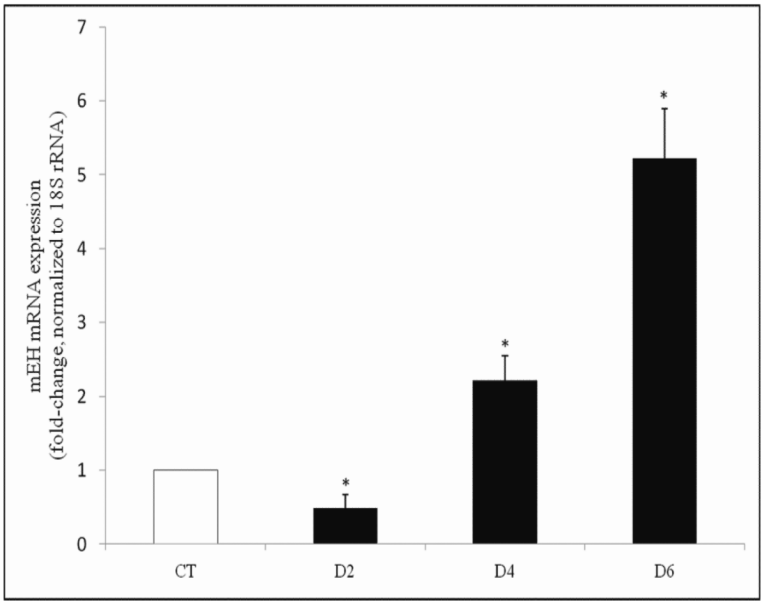

Figure 5. Effect of PI3K inhibition on *meh* mRNA expression

PND4 F344 rat ovaries were cultured in media containing vehicle control (CT), ± 20 μM LY294002 for 2 or 4 days. Total RNA was isolated and *meh* and *18S* rRNA levels were quantified by RT-PCR as described in methods. Values are expressed as mean fold change ± SE; n=3 (10 ovaries per pool). * $P < 0.05$; different from control.

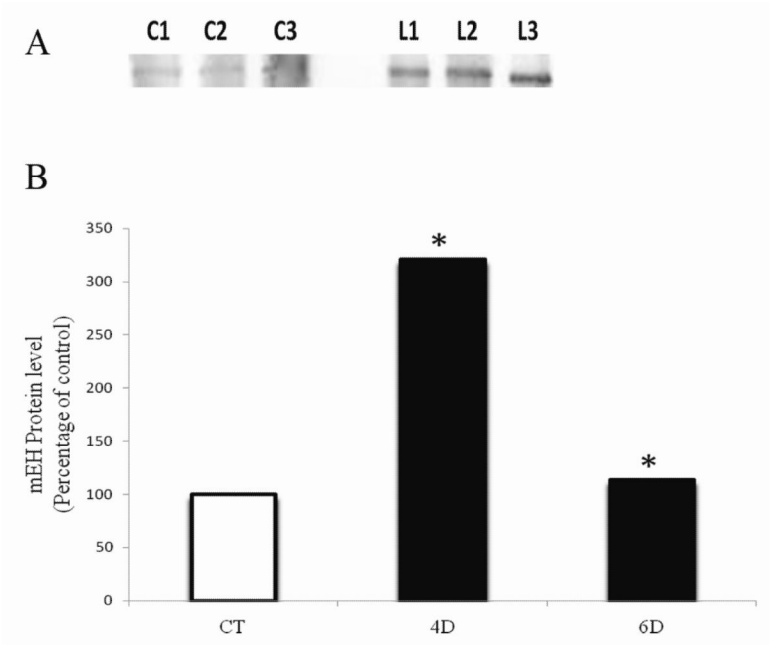

Figure 6. Temporal effect of PI3K inhibition on mEH protein

PND4 F344 rat ovaries were cultured in media containing vehicle control (CT), ± 20 μM LY294002 for 4 or 6 days. Total protein was isolated and Western blotting was performed to detect mEH protein. (A) Representative Western blot is shown on day 4; Control = C; LY294002 = L. (B) Values are expressed as a percentage of control mean ± SE; n=3 (10 ovaries per pool). * $P < 0.05$; different from control.