Toxicology and Applied Pharmacology 247 (2010) 71–75

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Dual protective role for Glutathione S-transferase class pi against VCD-induced ovotoxicity in the rat ovary ☆

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ARTICLE INFO

Article history:
Received 7 April 2010
Revised 28 May 2010
Accepted 2 June 2010
Available online 11 June 2010

Keywords:
4-vinylcyclohexene diepoxide
Ovotoxicity
Glutathione S-transferase pi

ABSTRACT

The occupational chemical 4-vinylcyclohexene diepoxide (VCD) selectively destroys ovarian small pre-antral follicles in rats and mice via apoptosis. Detoxification of VCD can occur through glutathione conjugation, catalyzed by glutathione *S*-transferase (GST) enzymes. Further, GST class pi (GSTp) can negatively regulate JNK activity through protein:protein interactions in extra-ovarian tissues. Dissociation of this protein complex in the face of chemical exposure releases the inhibition of pro-apoptotic JNK. Increased JNK activity during VCD-induced ovotoxicity has been shown in isolated ovarian small pre-antral follicles following *in vivo* dosing of rats (80 mg/kg/day; 15 days, i.p.). The present study investigated the pattern of ovarian GSTp expression during VCD exposure. Additionally, the effect of VCD on an ovarian GSTp:JNK protein complex was investigated. PND4 F344 rat ovaries were incubated in control medium ± VCD (30 μM) for 2–8 days. VCD increased ovarian *GSTp* mRNA ($P<0.05$) relative to control on d4-d8; whereas GSTp protein was increased ($P<0.05$) on d6–d8. A GSTp:JNK protein complex was detected by immunoprecipitation and Western blotting in ovarian tissues. Relative to control, the amount of GSTp-bound JNK was increased ($P=0.09$), while unbound JNK was decreased ($P<0.05$) on d6 of VCD exposure. The VCD-induced decrease in unbound JNK was preceded by a decrease in phosphorylated c-Jun which occurred on d4. These findings are in support of a possible dual protective role for GSTp in the rat ovary, consisting of metabolism of VCD and inhibition of JNK-initiated apoptosis.

Introduction

4-vinylcyclohexene (VCH) is an occupational chemical formed by dimerization of 1,3-butadiene and is produced as a by-product in the pesticide, rubber, plastic and flame retardant industries (Rappaport and Fraser, 1977). A metabolite of VCH, 4-vinylcyclohexene diepoxide (VCD) is used as an industrial diluent for epoxides (IARC, 1976). VCD is ovotoxic and specifically destroys small pre-antral follicles (primordial and primary) in ovaries of rats and mice (Kao et al., 1999; Smith et al, 1990) by enhancing the natural process of atresia in a time- and dose-dependent manner (apoptosis; Devine et al., 2002a; Springer et al., 1996a,b,c; Hu et al., 2001a,b, 2002). In the ovary the cytochrome P450 enzyme isoform CYP2E1 may play a role in converting VCH to the ovotoxic form, VCD (Cannady et al., 2003; Rajapaska et al., 2007). Ovarian detoxification of VCD to inactive metabolites is catalyzed through the action of microsomal epoxide hydrolase (mEH) and the glutathione *S*-transferase (GST) family of enzymes.

GST comprise a large family of enzymes that catalyze the conjugation of glutathione (GSH) to a variety of xenobiotics, allowing for their inactivation and more rapid excretion. There are a number of classes of GST enzymes, alpha, mu, omega, pi, sigma and theta, and each of these in turn contains a number of isoforms. The mouse ovary has been shown to synthesize GSH (Luderer et al., 2001), and form VCD-GSH adducts (Rajapaska, 2007). A previous study in cultured postnatal day (PND) 4 $B_6C_3F_1$ mouse ovaries showed no effect of VCD on mRNA encoding GST classes alpha, omega or theta (Keating et al., 2008). However, VCD exposure increased ($P<0.05$) mRNA encoding GST classes pi (GSTp) and mu (GSTm) on d4 by 1.55- and 1.7-fold, respectively. Yet this effect was reversed on d6 and d8. GSTp, but not GSTm protein was elevated by 47% after 8 days of VCD exposure. It was hypothesized that in mice, despite early up-regulation of GST, repeated VCD exposure eventually overwhelmed the induction of GST enzymes, thereby reducing its detoxification capacity during the onset of ovotoxicity (Keating et al., 2008).

In addition to its role in glutathione conjugation, the GST family has another possible indirect role in regulation of cell signaling. GSTp has been shown to form a protein complex with JNK and c-Jun (Adler et al., 1999), keeping JNK in an inactive state. This interaction prevents

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doi:10.1016/j.taap.2010.06.002

JNK mediated phosphorylation and activation of c-Jun (phosphorylated c-Jun; p-c-Jun). Following chemical exposure, this complex dissociates, relieving the inhibitory action of GSTp on JNK (Adler et al., 1999). A role for both JNK and p-c-Jun during VCD-induced ovotoxicity has been demonstrated. Following 15 days of daily dosing of rats with VCD (80 mg/kg; i.p.), relative to controls, both JNK (protein and activity) and p-c-Jun (protein) increased in isolated small pre-antral follicles (targeted by VCD; Hu et al., 2002).

An *in vitro* culture system has been developed using ovaries from PND4 rats (enriched in small pre-antral follicles) to examine *in vitro* effects of ovotoxicants without a metabolic influence from the liver (Devine et al., 2002b, 2004). Whether the GST and JNK pathways interact in the ovary as in other tissues is unknown. Therefore, the present study was designed to characterize in rats the association between VCD and GSTp mRNA and protein as relates to VCD-induced follicle loss using the *in vitro* ovary culture system. The formation of an ovarian GSTp and JNK-containing protein complex was also investigated.

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, Tris–HCl, sodium chloride, Tween-20, bovine serum albumin (BSA), ascorbic acid (Vitamin C), phosphatase inhibitor, protease inhibitor and transferrin were purchased from Sigma-Aldrich Inc. (St. Louis, MO). Dulbecco's Modified Eagle Medium: nutrient mixture F-12 (Ham) 1× (DMEM/Ham's F12), albumax, penicillin/streptomycin (5000 U/ml, 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, anti-p-c-Jun and anti-GSTp antibodies were purchased from Millipore (Bedford, MA). 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-JNK antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-ACTB antibody and agarose G beads were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Pierce Biotechnology (Rockford, IL).

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; 12 h light/12 h dark cycles). 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 described by Devine et al. (2002a). Briefly, PND4 female F344 rats were euthanized by CO_2 inhalation followed by decapitation. Each ovary was removed, the oviduct and excess tissue were trimmed, and it was placed on a piece of 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/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. Culture media was used as a solvent for VCD. The concentration of VCD used (30 μM) was determined previously to cause loss of primordial and small primary follicles following 6 days of exposure using the *in vitro* rat ovary culture system (Keating et al., 2009). For those cultures lasting more than 2 days, media were removed and fresh media and treatment were replaced every 2 days.

RNA isolation. Following 2, 4, 6 or 8 days of *in vitro* culture, ovaries treated with 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 then centrifuged at 14,000 rpm for 2 min. The resulting flow-through 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 = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE).

First strand cDNA synthesis and real-time polymerase chain reaction (PCR). Total RNA (0.5 μg) was reverse transcribed into cDNA utilizing the Superscript III One-Step RT-PCR System. cDNA 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 as described in Keating et al. (2008). The cycling program consisted of a 15 min hold at 95 °C and 45 cycles of: denaturing at 95 °C for 15 s, annealing at 58 °C for 15 s, and extension at 72 °C for 20 s 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 control and VCD treated ovaries. Therefore, each sample was normalized to ACTB before quantification using the $2^{-\Delta\Delta Ct}$ method.

Protein isolation. Pools of whole ovarian protein (10–20 ovaries/pool) homogenates were prepared from cultured ovaries via homogenization in tissue lysis buffer containing protease and phosphatase inhibitors as previously described (Thompson et al., 2005). For immunoprecipitation protein isolation, SDS was omitted from the tissue lysis buffer. 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 BCA protocol on a 96-well assay plate. Emission absorbance was 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.

Protein immunoprecipitation. Ovarian protein (100 μg) was incubated overnight at 4 °C with 20 μl of anti-GSTp antibody. Protein G agarose beads were washed, added to the protein-antibody mixture and incubated at 4 °C for 3 hours with turning. This mixture was centrifuged at 10,000 rpm, and the supernatant (unbound fraction) was removed for Western blotting. The beads were washed three times with tissue lysis buffer (500 μl). Laemmli sample buffer (20 μl) was added and beads incubated at 95 °C for 10 min. The beads were centrifuged and 10 μl supernatant (bound fraction) was used for Western blotting.

Western blot analysis. SDS-PAGE (10%) was used to separate protein homogenates ($n = 3$; 10 μg total protein or 10 μl unbound or bound immunoprecipitation fractions) and subsequently transferred onto nitrocellulose membranes as previously described (Thompson et