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Involvement of a volatile metabolite during phosphoramide mustard-induced ovotoxicity

Jill A. Madden [a], Patricia B. Hoyer [b], Patrick J. Devine [c,1], Aileen F. Keating [a,b,*]

[a] Department of Animal Science, Iowa State University, Ames, IA 50011, USA
[b] Department of Physiology, University of Arizona, Tucson, AZ 85724, USA
[c] INRS—Institut Armand-Frappier Research Centre, University of Quebec, Laval, QC H7V 1B7, Canada

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ABSTRACT

The finite ovarian follicle reserve can be negatively impacted by exposure to chemicals including the anti-neoplastic agent, cyclophosphamide (CPA). CPA requires bioactivation to phosphoramide mustard (PM) to elicit its therapeutic effects however; in addition to being the tumor-targeting metabolite, PM is also ovotoxic. In addition, PM can break down to a cytotoxic, volatile metabolite, chloroethylaziridine (CEZ). The aim of this study was initially to characterize PM-induced ovotoxicity in growing follicles. Using PND4 Fisher 344 rats, ovaries were cultured for 4 days before being exposed once to PM (10 or 30 μM). Following eight additional days in culture, relative to control (1% DMSO), PM had no impact on primordial, small primary or large primary follicle number, but both PM concentrations induced secondary follicle depletion ($P < 0.05$). Interestingly, a reduction in follicle number in the control-treated ovaries was observed. Thus, the involvement of a volatile, cytotoxic PM metabolite (VC) in PM-induced ovotoxicity was explored in cultured rat ovaries, with control ovaries physically separated from PM-treated ovaries during culture. Direct PM (60 μM) exposure destroyed all stage follicles after 4 days ($P < 0.05$). VC from nearby wells depleted primordial follicles after 4 days ($P < 0.05$), temporarily reduced secondary follicle number after 2 days, and did not impact other stage follicles at any other time point. VC was determined to spontaneously liberate from PM, which could contribute to degradation of PM during storage. Taken together, this study demonstrates that PM and VC are ovotoxicants, with different follicular targets, and that the VC may be a major player during PM-induced ovotoxicity observed in cancer survivors.

Introduction

Cyclophosphamide (CPA) is used, alone or in combination, to treat an array of cancers and autoimmune disorders, and elicits numerous undesirable health side effects including alopecia, nausea, immunosuppression and infertility (Fraiser et al., 1991). As a prodrug, CPA requires bioactivation to the metabolite phosphoramide mustard (PM) in order to induce anti-neoplastic effects (Shulman-Roskes et al., 1998). Bioactivation of CPA to PM occurs through a cascade of reactions (Fig. 1) that are initiated primarily by the hepatic cytochrome P450 enzyme (CYP) isoforms 2B and 3A (Philip et al., 1999). These CYPs hydroxylate CPA to form 4-hydroxycyclophosphamide, which is non-enzymatically transformed to the open-ring metabolite, aldophosphamide. Aldophosphamide fragments, producing the compounds acrolein and PM (Ludeman, 1999).

Interestingly, PM can further transform into a volatile, cytotoxic compound chloroethylaziridine (CEZ; Lu and Chan, 1996; Rauen and Norpoth, 1968). CEZ was first identified as a volatile metabolite of CPA in 1968, when approximately 2% of the CPA administered was shown to be exhaled as CEZ from the lungs of exposed rats (Rauen and Norpoth, 1968). CEZ was also found to be responsible for the airborne cytotoxicity, observed in untreated cells cultured adjacent to cells exposed to CEZ-producing compounds, termed the "neighboring well effect" (Flowers et al., 2000). Although the generation of CEZ has been demonstrated both *in vivo* and *in vitro*, determining the relative contribution of CEZ to CPA-induced cytotoxicity has proven difficult due to the compound's volatility and the instability of its precursor, PM. CEZ plasma concentrations were shown to peak 5 min after intravenous PM administration in rats (Lu and Chan, 2006). An additional study demonstrated that following complete degradation of PM in solution, 85% of the solution's cytotoxicity remained due to the generation and continued presence of CEZ (Chan et al., 1994). Taken together, these studies support that CEZ is a major degradation product of CPA/PM and suggest that CEZ may play a key role in the toxicity of CPA.

CPA-induced side effects are of growing concern as cancer survival rates continue to improve. In particular, increased cancer survival rates have resulted in a greater number of female cancer survivors affected by CPA-induced infertility (Linet et al., 1999; Pulte et al., 2008; Sklar et al., 2006). Female fertility is dependent on the quality of

* Corresponding author at: Department of Animal Science, Iowa State University, Ames, IA 50011, USA. Fax: +1 515 294 4471.
E-mail address: akeating@iastate.edu (A.F. Keating).
[1] Current affiliation address: Novartis Institutes for Biomedical Research Inc., Cambridge, MA 02139, USA.

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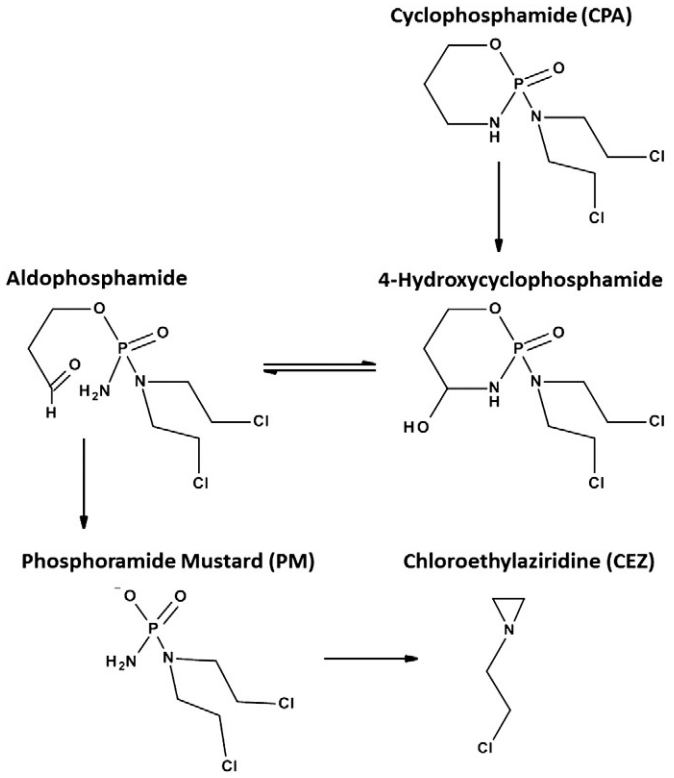

Fig. 1. CPA metabolism. CPA metabolism is initiated by CYP2B and CYP3A to form 4-hydroxycyclophosphamide, which is non-enzymatically transformed to aldophosphamide. Fragmentation of aldophosphamide forms PM and further partitioning of PM forms CEZ. Adapted from Hong and Chan, 2006.

oocytes, which are maintained within the ovary as follicular structures, consisting of a meiotically-arrested oocyte surrounded by granulosa cells. The ovarian follicular reserve is established at birth and progressively declines until menopause or ovarian failure, the point at which no follicles remain (Hirshfield, 1991). Exposure to chemicals, including CPA, accelerates follicular death and subsequent decline of the follicular reserve leading to premature ovarian failure (POF), thus causing permanent infertility (Plowchalk and Mattison, 1991). Aside from sustaining fertility, the survival of these follicles is important to overall female health; POF increases the risk of various health conditions including osteoporosis and heart disease (Greendale et al., 1999).

CPA-induced infertility is attributed to the generation of PM because, in addition to being an anti-neoplastic metabolite, PM is also recognized as an ovotoxic CPA metabolite (Desmeules and Devine, 2006; Plowchalk and Mattison, 1991). However, CEZ has also been suggested to be ovotoxic due to observed loss of primordial follicles induced by a volatile breakdown product of PM in a neonatal rat ovarian culture model (Desmeules and Devine, 2006). Therefore, in addition to PM, CEZ may be at least partially responsible for both the anti-neoplastic and ovotoxic properties of CPA. The purpose of the current study was initially to determine the impact of acute exposure to PM on large growing follicles, which led to the characterization of the ovotoxicity of a volatile compound (VC), presumably CEZ, liberated from PM using a neonatal rat ovary culture system. Specifically, the temporal pattern of PM- and VC-induced follicle loss was characterized, the requirement of ovarian tissue for VC liberation was determined and the impact of storage on PM-induced ovotoxicity at −20 °C was evaluated.

Materials and methods

Reagents. Bovine serum albumin (BSA), ascorbic acid, transferrin, formaldehyde, dimethyl sulfoxide (DMSO) 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 (5000 U/ml) and Hank's Balanced Salt Solution (without $CaCl_2$, Mg Cl_2, or $MgSO_4$) were obtained from Invitrogen Co. (Grand Island, NY). Millicell-CM filter inserts and 48-well cell culture plates were obtained from Millipore (Billerica, MA) and Corning Inc. (Corning, NY), respectively. Phosphoramide mustard was obtained from the National Institutes of Health National Cancer Institute (Bethesda, MA).

Animals. Fisher 344 (F344) rats were housed one per plastic cage and maintained in a controlled environment (22 ± 2 °C; 12 h light/12 h dark cycles). The animals were provided a standard diet with *ad libitum* access to food and water and allowed to give birth. The University of Arizona and Iowa State University Institutional Animal Care and Use Committee approved all experimental procedures.

Ex vivo ovarian cultures. Ovaries were collected from female postnatal day (PND) 4 F344 rats and cultured as described by Devine et al., 2002. Ovaries were removed, trimmed of oviduct and other excess tissue, and placed onto a Millicell-CM membrane floating on 250 μl of previously 37 °C equilibrated 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. A drop of medium was placed on top of each ovary to prevent dehydration and maintained at 37 °C and 5% CO_2.

Effect of single exposure PM on growing follicles. Ovaries (n = 3/treatment) were cultured for four days to allow large primary and secondary follicles to develop in culture before being treated once with vehicle control media (1% DMSO), PM (10 μM or 30 μM) and maintained in culture for an additional eight days. These concentrations were based on those previously described (Petrillo et al., 2011).

Ovotoxicity time course. Ovaries (n = 3–4/treatment) were treated on alternate days with vehicle control DMSO (1%), PM (60 μM) or VC for 2, 4 or 6 days. These PM concentrations were chosen to achieve a phenotypic endpoint of approximately 50% primordial follicle loss and to achieve sufficient VC liberation for ovotoxicity evaluation. All PM-treated ovaries were maintained in a separate incubator from the control-treated ovaries (CT). VC-exposed ovaries were cultured in control media in the same incubator as the PM treated ovaries, but on separate plates from the PM samples, thus the ovaries were exposed to the volatile metabolite liberated from PM-treated wells.

Evaluation of ovarian-required VC generation. Ovaries (n = 4) were cultured for six days in control media adjacent to wells that were treated on alternate days with PM (60 μM) but did not contain an ovary (Fig. 2).

Evaluation of PM degradation during storage. Cultured ovaries (n = 4) were treated on alternate days with freshly suspended PM ("New") or with previously suspended PM, which had been stored for two years in DMSO at −20 °C ("Old"). Ovaries were cultured for six days in separate incubators to reduce VC-induced ovotoxicity as a confounding factor.

Histological evaluation of follicle numbers. Following treatment, ovaries were placed in 4% paraformaldehyde fixative for 2 h, washed and stored in 70% ethanol, paraffin embedded, and serially sectioned (5 μM) at the histology laboratory in the Department of Veterinary Pathology (Iowa State University). Every 6th section was mounted and stained with hematoxylin and eosin. Healthy oocyte-containing follicles were identified and counted in every 6th section. Unhealthy follicles were identified from healthy follicles by the appearance of pyknotic bodies and intense eosinophilic staining of oocytes. Healthy follicles were classified and enumerated according to Flaws et al., 1994. Slides were blinded to prevent counting bias.