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Glutathione deficiency sensitizes cultured embryonic mouse ovaries to benzo[*a*]pyrene-induced germ cell apoptosis

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ABSTRACT

Mice lacking the modifier subunit of glutamate cysteine ligase (*Gclm*), the rate-limiting enzyme in glutathione (GSH) synthesis, have decreased tissue GSH. We previously showed that *Gclm*−/− embryos have increased sensitivity to the prenatal *in vivo* ovarian toxicity of the polycyclic aromatic hydrocarbon benzo[*a*]pyrene (BaP) compared with *Gclm*+/+ littermates. We also showed that BaP-induced germ cell death in cultured wild type embryonic ovaries is caspase-dependent. Here, we hypothesized that GSH deficiency increases sensitivity of cultured embryonic ovaries to BaP-induced germ cell death. 13.5 days post coitum (dpc) embryonic ovaries of all *Gclm* genotypes were fixed immediately or cultured for 24 h in media supplemented with DMSO vehicle or 500 ng/ml BaP. The percentage of activated caspase-3 positive germ cells varied significantly among groups. Within each genotype, DMSO and BaP-treated groups had increased germ cell caspase-3 activation compared to uncultured. *Gclm*+/− ovaries had significantly increased caspase-3 activation with BaP treatment compared to DMSO, and caspase-3 activation increased non-significantly in *Gclm*−/− ovaries treated with BaP compared to DMSO. There was no statistically significant effect of BaP treatment on germ cell numbers at 24 h, consistent with our prior observations in wild type ovaries, but *Gclm*−/− ovaries in both cultured groups had lower germ cell numbers than *Gclm*+/+ ovaries. There were no statistically significant BaP-treatment or genotype-related differences among groups in lipid peroxidation and germ cell proliferation. These data indicate that *Gclm* heterozygous or homozygous deletion sensitizes embryonic ovaries to BaP- and tissue culture-induced germ cell apoptosis.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs), such as benzo[*a*]pyrene (BaP), are ubiquitous environmental toxicants generated during incomplete combustion of organic materials, such as wood, fossil fuels, tobacco, and foods (ATSDR, 1995). PAHs generally require metabolism to reactive metabolites to exert toxicity (Xue and Warshawsky, 2005). Metabolism of BaP and other PAHs results in the formation of DNA adduct-forming, mutagenic metabolites (Xue and Warshawsky, 2005), and several of the metabolic pathways also generate reactive oxygen species (ROS) (Penning et al., 1996; Wells et al., 2009). ROS play a role in maintaining physiological functions as intracellular signaling molecules and in host defense systems, but excessive production of ROS can lead to the disruption of intracellular redox balance, a state of oxidative stress, damage to cell components, and ultimately cell death (Jones, 2006).

The tripeptide glutathione (GSH) is the most abundant intracellular nonprotein thiol, with intracellular concentrations in the millimolar range. GSH has numerous intracellular functions, not only in antioxidant defenses, but also in many metabolic processes (Shan et al., 1990; Anderson and Luo, 1998). GSH can scavenge free radicals directly, participate in the reduction of hydrogen peroxide and lipid peroxides as a cofactor for glutathione peroxidases and peroxiredoxin, and detoxify electrophilic toxicants as a cofactor for glutathione transferases (Shan et al., 1990; Anderson and Luo, 1998; Dalton et al., 2004). Our work has shown that GSH is important in protecting mature ovarian follicles against apoptotic stimuli, including PAHs (Tsai-Turton and Luderer, 2006; Tsai-Turton et al., 2007). GSH is synthesized in two ATP-dependent reactions. The first, rate-limiting reaction is catalyzed by GCL, a heterodimer composed of a catalytic (GCLC) and a modifier (GCLM) subunit (Griffith and Mulcahy, 1999; Franklin et al., 2009). GCLC is responsible for the catalytic activity of the enzyme, while binding of GCLM to GCLC decreases the Michaelis constant for the substrates glutamate and ATP and increases the inhibitory constant for

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GSH (Griffith and Mulcahy, 1999; Franklin et al., 2009). *Gclc*−/− mice die during embryonic development (Dalton et al., 2000), whereas *Gclm*−/− mice survive and reproduce, despite greatly decreased tissue levels of GSH (Yang et al., 2002; McConnachie et al., 2007).

Primordial germ cell (PGC) migration to the developing gonad, mitotic proliferation during migration and after arrival at the gonad, and the transition from mitosis to meiosis are critical prenatal developmental events that determine the size of the ovarian follicle reserve after birth. Disruption of any of these events can result in decreased ovarian reserve, resulting in premature ovarian senescence. PGCs in the mouse first emerge in the yolk sac at 7.25 days postcoitum (dpc), then migrate to the gonadal ridge (the future ovary), arriving at 10.5 dpc (McLaren, 2003; Pepling, 2006). During migration and after arriving in the ovary, PGCs actively proliferate until 13.5 dpc when they begin to enter meiosis (McLaren, 2003; Pepling, 2006). Now called oocytes, they progress through prophase I of meiosis and arrest in the diplotene stage beginning at 17.5 dpc (Pepling, 2006). In humans, primordial germ cells arrive at the gonad around gestational week 3, begin to enter meiosis during week 10, and are enclosed in primordial follicles by the end of the second trimester. Establishment of this primordial follicle pool is vital for fertility and reproductive health in adulthood. The developing ovary is an important target for PAHs, including BaP. Maternal smoking, a primary route of human prenatal exposure to BaP, disturbs human ovarian development and endocrine signaling (Fowler et al., 2014), decreases fecundity (Weinberg et al., 1989) and is associated with earlier onset of menopause in daughters (Strohsnitter et al., 2008). Our previous studies showed that exposure of pregnant mice to BaP from 6.5 to 15.5 dpc depletes germ cells, resulting in decreased ovarian reserve, decreased fertility, and epithelial ovarian tumors in the F1 female offspring (Lim et al., 2013; Luderer et al., 2017). Moreover, we showed that BaP treatment of cultured wild type embryonic day 13.5 ovaries depletes germ cells *via* induction of caspase-dependent, apoptotic death of these cells (Lim et al., 2016).

Our prior work showed that GSH-deficient *Gclm*−/− female mice have increased sensitivity to destruction of germ cells by *in vivo* transplacental exposure to BaP compared with *Gclm*+/+ littermates (Lim et al., 2013), indicating that GSH is protective against the prenatal ovarian toxicity of BaP. Here, we tested the hypotheses that *Gclm* deficient embryonic ovaries are more sensitive to induction of oxidative damage and germ cell apoptosis in response to the stress of culture and BaP treatment than *Gclm*+/+ ovaries.

2. Material and methods

2.1. Animals

Gclm−/− mice were generated by disrupting the *Gclm* gene by replacing exon 1 with a β-galactosidase/neomycin phosphotransferase fusion gene and were backcrossed onto a C57BL/6 J genetic background (Giordano et al., 2006; McConnachie et al., 2007). Mice for these experiments were bred in our colony and housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility, with free access to deionized water and laboratory chow (Harlan Teklad 2919) on a 14:10 h light-dark cycle. Temperature was maintained at 69–75 °F. The experimental protocols were carried out in accordance with the Guide for the Care and Use of Laboratory Animals (NRC, 2011) and were approved by the Institutional Animal Care and Use Committee at the University of California Irvine. 10-week old *Gclm*+/− females were mated with *Gclm*+/+ or *Gclm*−/− males on the evening of proestrus, determined by vaginal cytology, and the morning after overnight mating was considered 0.5 days postcoitum (dpc) if a vaginal plug was found. Pregnant mice were sacrificed by CO_2 euthanasia on 13.5 dpc, and the embryos were quickly removed from the uterus. Embryos were dissected using a stereomicroscope, and the sex was determined by the morphology of the gonads. DNA was extracted from embryo tails for *Gclm* genotyping as previously described (Giordano et al., 2006).

2.2. Embryonic ovary culture and BaP treatment

Ovaries from 13.5 dpc embryos were dissected with mesonephros intact and either fixed immediately (0 h) or cultured as described previously (Lim et al., 2016) for 24 h in media with 0.005% dimethyl sulfoxide (DMSO) alone or with 500 ng/ml BaP (Supelco, Bellefonte, PA, USA) in Dulbecco's modified Eagle's medium/Ham F12 (1:1) (Gibco, Grand Island, NY, USA) supplemented with 0.1% bovine serum albumin, 100 μg/ml streptomycin and 100 IU/ml penicillin G. The two ovaries from the same embryo were always assigned to different treatment groups. Ovaries from embryos of 4 to 8 different pregnant dams were used or each of the 9 experimental groups (3 *Gclm* genotypes times 3 experimental conditions). We chose the concentration and duration of culture based on our prior study in which we observed statistically significant caspase-9 and -3 activation at 24 h and decreased germ cell numbers at 48 h in wild type embryonic ovaries exposed to 1000 ng/ml, but not 500 ng/ml, BaP (Lim et al., 2016). We chose a concentration that did not induce apoptosis in wild type ovaries so that we would be able to detect increased sensitivity of the *Gclm* deficient ovaries. A fresh aliquot of stock solution (20 mg/ml) was used to make the BaP treatment media for each experimental run. Ovaries were placed on 0.4 μm Millicell-CM Biopore membranes (Millipore, Billerica, MA, USA) floating on 400 μl culture medium in tissue culture dishes and cultured at 37 °C in a humidified atmosphere containing 95% air and 5% carbon dioxide. At the end of the culture, ovaries were fixed in Bouin's solution overnight at 4 °C and embedded in OCT before being stored at −80 °C. The embedded ovaries were sectioned at 5 μm for immunostaining.

2.3. Germ cell counting

Complete serial sections were cut for every ovary and mounted so that there were four sets of slides with four sections per slide, which were separated by 3 intervening sections. One complete set of slides, containing every 4th section through the entire ovary was immunostained with TRA98 antibody for germ cell counts, and the other sets were used for immunostaining with other antibodies as described below. We performed immunofluorescence assays for germ cell-specific antigen (TRA98) using a rat anti-TRA98 monoclonal IgG antibody (1:200; Abcam #82527, Cambridge, MA, USA) as previously described (Lim et al., 2016). After incubation with Alexa 488 goat anti-rabbit IgG, the germ cells were identified by their green fluorescence and distinctive morphology with large size and spherical shape. We counted germ cells in every fourth section and multiplied the sum of the values obtained for the observed sections of one ovary by 4 to obtain a total count of germ cells per ovary. All counts were carried out blind to treatment using ImageJ software for counting (National Institutes of Health, Bethesda, MD, USA) on images captured using a Retiga 2000R digital camera with an Olympus BX60 microscope equipped with fluorescence filters.

2.4. Immunohistochemistry

We previously validated the antibodies against the mitotic marker Ki67, lipid peroxidation marker 4-hydroxynonenal (4-HNE) and apoptosis marker cleaved caspase-3 in mouse embryonic and adult ovaries (Lim et al., 2015; Lim et al., 2016). Slides were thawed and heated for 15 min at 95 °C in a 10 mM citrate buffer (pH 6.0) for antigen retrieval. The primary antibodies, rabbit anti-cleaved caspase-3 Asp 175 (1:100; Cell Signaling #9664, Beverly, MA, USA), rabbit anti-4-HNE (1:500; Alpha diagnostic #HNE11-S, San Antonio, TX, USA), and rabbit anti-Ki67 (1:500; Abcam #15580), were detected using biotinylated goat anti-rabbit secondary antibodies in 5% normal goat serum and avidin–biotin–peroxidase complex (Vectastain Elite ABC kit; Vector