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Effects of deletion of the transcription factor *Nrf2* and benzo [*a*]pyrene treatment on ovarian follicles and ovarian surface epithelial cells in mice

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ABSTRACT

Polycyclic aromatic hydrocarbons, like benzo[*a*]pyrene (BaP), are ubiquitous environmental pollutants and potent ovarian toxicants. The transcription factor NRF2 is an important regulator of the cellular response to electrophilic toxicants like BaP and to oxidative stress. NRF2 regulates transcription of genes involved in the detoxification of reactive metabolites of BaP and reactive oxygen species. We therefore hypothesized that *Nrf2*−/− mice have accelerated ovarian aging and increased sensitivity to the ovarian toxicity of BaP. A single injection of BaP dose-dependently depleted ovarian follicles in *Nrf2*+/+ and *Nrf2*−/− mice, but the effects of BaP were not enhanced in the absence of *Nrf2*. Similarly, *Nrf2*−/− mice did not have increased ovarian BaP DNA adduct formation compared to *Nrf2*+/+ mice. Ovarian follicle numbers did not differ between peripubertal *Nrf2*−/− and *Nrf2*+/+ mice, but by middle age, *Nrf2*−/− mice had significantly fewer primordial follicles than *Nrf2*+/+ mice, consistent with accelerated ovarian aging.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous environmental pollutants, which are formed during the incomplete combustion of organic materials. Tobacco smoke is rich in PAHs [1]. Inhalation exposure to PAHs also occurs in non-smokers, due to exposure to second hand tobacco smoke and to air pollution. PAHs in ambient urban air are more than 10-fold higher than in rural air [2,3]. Grilling or smoking of food also generates PAHs, and frequent consumption of grilled or smoked foods is another major source of PAH exposure [2,3].

Women who smoke have decreased per menstrual cycle probability of pregnancy compared to women who do not smoke [4–6] . The onset of menopause occurs several years earlier in women who smoke [7,8]. The adverse ovarian effects of smoking may be caused by PAHs present in tobacco smoke. The PAHs dimethylbenzanthracene (DMBA), 3-methylcholanthrene (3MC), and benzo[*a*]pyrene (BaP) all destroy follicles in mice after single high doses [9–12] and after repeated low doses [13] . DMBA also destroyed ovarian follicles in human ovarian explants, which were implanted subcutaneously in mice [14]. Prenatal and postnatal exposure to PAHs also causes ovarian tumors later in life [15,16]. We recently showed that prenatal exposure to BaP causes ovarian tumors, which exhibit high levels of the epithelial marker cytokeratin by immunostaining [17]. The latter results suggest that PAH-induced ovarian tumors may arise from the ovarian surface epithelial (OSE) cells from which most malignant ovarian tumors in women are believed to arise [18,19]. BaP has been shown to induce proliferation of mammary tumor cells in vitro [20–22], but effects of BaP on OSE cell proliferation in vivo have not been examined.

PAHs are oxidized by microsomal cytochrome P450 enzymes (chiefly P450s 1A1 and 1B1) to epoxides, which are converted to diols by microsomal epoxide hydrolase (*Ephx1*) [23,24]. Diols can undergo further oxidation by P450 enzymes to mutagenic and ovotoxic diol epoxides [23,25]. Alternatively, they can be metabolized by diol dehydrogenases, ultimately yielding catechols, which can undergo two sequential one-electron oxidations to *o*-semiquinone and then to *o*-quinone, generating hydrogen peroxide and superoxide anion radical [24]. The *o*-quinone can be reduced back to the semiquinone or catechol, resulting in redox cycles. All of the enzymes required to activate PAHs are present in the ovary [26–32].

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Glutathione-*S*-transferase (GST)-catalyzed conjugation with glutathione (GSH) is an important Phase II detoxification mechanism for the diol epoxide, diol, and quinone metabolites of PAHs [33], and GSH also reduces and detoxifies reactive oxygen species (ROS) via direct reactions or as a cofactor for glutathione peroxidases.

The Cap 'n Collar basic leucine zipper transcription factor nuclear factor 2 erythroid related factor 2 (NRF2) regulates transcription of GSTs, the enzymes of GSH synthesis, superoxide dismutase, NADPH quinone oxidoreductase, and UDP-glucuronosyltransferase, which are important in detoxifying PAH metabolites and ROS, and of *Ephx1*, which is required to generate ovotoxic metabolites of PAHs [34–36]. *Nrf2* null mice are more susceptible than wild type mice to chemical toxicity, including cancer induction by the PAH BaP [37], ovarian toxicity by vinylcyclohexene diepoxide [38], and liver toxicity by acetaminophen [39] . They are also more susceptible to autoimmune diseases [40,41] than wild type mice. *Nrf2*−/− mice also exhibit increased oxidative stress compared to *Nrf2+/+* mice [42]. We previously reported that young male *Nrf2*−/− mice had normal spermatogenesis, but developed age-related defects in spermatogenesis compared to wild type littermates, which was associated with increased testicular oxidative lipid damage [43]. We and others have shown that oxidative stress is associated with normal ovarian aging [44–46] and that deletion of the antioxidant gene *Gclm* causes accelerated ovarian aging [47].

Herein we report on the results of experiments testing the hypotheses that (1) *Nrf2*−/− mice are more sensitive to ovarian DNA damage, apoptosis, and follicle destruction by BaP than *Nrf2+/+* mice due to their decreased ability to detoxify reactive metabolites of BaP; (2) BaP stimulates OSE cell proliferation and this occurs to a greater extent in *Nrf2*−/− ovaries than in *Nrf2+/+* ovaries; (3) *Nrf2* deletion accelerates the age-related decline in ovarian follicle numbers.

2. Methods

2.1. Materials

All chemicals and reagents were purchased from Sigma–Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA), unless otherwise noted.

2.2. Animals

Nrf2 null mice were generated by disrupting the *Nrf2* gene by homologous recombination in embryonic stem cells, using a targeting vector that results in deletion of part of exon 4 and all of exon 5, replacing them with a *LacZ* reporter gene [48] . Mice for these experiments were generated in our breeding colony by mating *Nrf2+/−* males with *Nrf2+/−* females. *Nrf2+/−* breeder mice had been backcrossed 8 times onto a C57BL/6Crl genetic background. Genotyping of tail snip DNA by PCR was carried out as described [48]. The mice were housed in an American Association for the Accreditation of Laboratory Animal Care-accredited facility, with free access to deionized water and laboratory chow (Prolab RMH 2500), on a 14:10 h light–dark cycle. Temperature was maintained at 21–24 °C. Experimental females were group-housed with their female littermates after weaning (up to 5 mice per cage). The experimental protocols were carried out in accordance with the *Guide for the Care and Use of Laboratory Animals* [49] and were approved by the Institutional Animal Care and Use Committee at the University of California Irvine.

2.3. Experimental protocol—ovarian effects of BaP treatment in *Nrf2+/+* and *Nrf2*−/− mice

To investigate the effects of lack *Nrf2* on the ovarian sensitivity to BaP, 28-day old *Nrf2*−/− and *Nrf2+/+* female mice were injected intraperitoneally with 0, 2, or 50 mg/kg BaP dissolved in sesame oil ($N = 5$ to 9/group). These doses were chosen based on a prior study, which showed that a single dose of 50 mg/kg depleted primordial follicles by 56%, while a 5 mg/kg dose depleted primordial follicles by 18% [12]. Seven days later at 35 days of age, mice were euthanized using carbon dioxide inhalation. One ovary per animal was randomly chosen for fixation in Bouin's fixative for 24 h, followed by 4 washes in 50% ethanol, and storage in 70% ethanol. The other ovary was fixed in 4% paraformaldehyde (PFA) in PBS for 1 h, then cryoprotected in 15% sucrose in PBS for 4 h prior to being embedded in Tissue-Tek OCT (Sakura Finetek, Torrance, CA). The embedded ovaries were serially sectioned at 10 μm for immunohistochemistry and terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL). Another set of mice was treated identically with 0 or 50 mg/kg BaP, and both ovaries were snap frozen on dry ice for subsequent DNA extraction for ^{32}P-postlabeling to detect BaP-related DNA adducts.

2.4. GSH assays

For GSH assays, ovaries from 6 *Nrf2+/+* and 7 *Nrf2*−/− 18–22 day old mice were homogenized in 20 mM Tris, 1 mM EDTA, 250 mM sucrose, 2 mM L-serine, 20 mM boric acid (TES-SB). After removal of aliquots for protein assay, supernatants were acidified with one quarter volume 5% sulfosalicylic acid for GSH assays [50]. Total GSH was measured using a modification of an enzymatic recycling assay developed by Griffith [50–52]. Triplicates of samples or standards were combined with 33 μl metal free water and incubated for 10 min at 30 °C. The samples were mixed with 140 μl of 0.3 mM NADPH, 20 μl of 6 mM DTNB (5,5′-dithiobis) (2-nitrobenzoic acid), and 2 μl of 50 units/mL GSH reductase. The rate of thiobis(2-nitrobenzoic acid) (TNB) formation from DTNB is proportional to the total GSH concentration in each sample. TNB formation was monitored by measuring the absorbance at 412 nm for 5 min every 10 s using a VersaMax microplate spectrophotometer (Molecular Devices, Sunnyvale, CA). The concentrations of total GSH in the samples were calculated from a standard curve generated from the slopes of the standards.

2.5. Immunoblotting for GCLC, GCLM, SOD2 in ovarian extracts

Ovaries from 34 to 36 day old *Gclm*−/− and *Gclm+/+* littermates were homogenized in RIPA buffer (PBS, 0.5% sodium deoxycholate, 0.1% SDS, 1% NP40) with protease inhibitors (Pefablock, TLCK, pepstatin, aprotonin, leupeptin) using a Kontes handheld dounce homogenizer (Kimble-Chase, Vineland, NJ) on ice. Protein concentration was determined by Pierce BCA (bicinchoninic acid) Assay. Twenty to forty micrograms of each protein sample were separated by SDS-PAGE, transferred to polyvinylidine difluoride membranes, blocked in 5% nonfat milk in phosphate buffered saline with 0.1% Tween-20, incubated with primary antibodies diluted in 3% nonfat dry milk, 3% BSA, 1% ovalbumin, 1% normal goat serum, 0.1% sodium azide, and then visualized with ECL chemiluminescence (GE Healthcare Lifesciences, Piscataway, New Jersey). GCL subunit antibodies were raised against ovalbumin conjugates and were a kind gift of Dr. Terrance J. Kavanagh, University of Washington [53]. GCLC subunit antibodies were used at a dilution of 1:40,000 and GCLM at 1:20,000. Antibody directed against manganese containing superoxide dismutase (SOD2) was purchased from Stressgen (#SOD-110, Ann Arbor, MI) and used at a 1:5000 dilution. All immunoblots were re-probed with monoclonal anti-β-actin mouse