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Impact of 7,12-dimethylbenz[a]anthracene exposure on connexin gap junction proteins in cultured rat ovaries

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ABSTRACT

7,12-Dimethylbenz[a]anthracene (DMBA) destroys ovarian follicles in a concentration-dependent manner. The impact of DMBA on connexin (CX) proteins that mediate communication between follicular cell types along with pro-apoptotic factors *p53* and *Bax* were investigated. Postnatal day (PND) 4 Fisher 344 rat ovaries were cultured for 4 days in vehicle medium (1% DMSO) followed by a single exposure to vehicle control (1% DMSO) or DMBA (12.5 nM or 75 nM) and cultured for 4 or 8 days. RT-PCR was performed to quantify *Cx37*, *Cx43*, *p53* and *Bax* mRNA level. Western blotting and immunofluorescence staining were performed to determine CX37 or CX43 level and/or localization. *Cx37* mRNA and protein increased ($P < 0.05$) at 4 days of 12.5 nM DMBA exposure. Relative to vehicle control-treated ovaries, mRNA encoding *Cx43* decreased ($P < 0.05$) but CX43 protein increased ($P < 0.05$) at 4 days by both DMBA exposures. mRNA expression of pro-apoptotic *p53* was decreased ($P < 0.05$) but no changes in *Bax* expression were observed after 4 days of DMBA exposures. In contrast, after 8 days, DMBA decreased *Cx37* and *Cx43* mRNA and protein but increased both *p53* and *Bax* mRNA levels. CX43 protein was located between granulosa cells, while CX37 was located at the oocyte cell surface of all follicle stages. These findings support that DMBA exposure impacts ovarian *Cx37* and *Cx43* mRNA and protein prior to both observed changes in pro-apoptotic *p53* and *Bax* and follicle loss. It is possible that such interference in follicular cell communication is detrimental to follicle viability, and may play a role in DMBA-induced follicular atresia.

Introduction

The female gamete, the oocyte, is encased in a follicular structure surrounded by granulosa cells and, as the follicle matures, also by theca cells. Primordial follicles are maintained in a dormant state until activation into the growing follicular pool (Hirshfield, 1991). In women, nearly 99% of ovarian follicles undergo degenerative changes by a process known as atresia (Hirshfield, 1991) and ovarian senescence (menopause) occurs when the finite pool of primordial follicles has become exhausted (Broekmans et al., 2007; Hansen et al., 2008; Mattison and Nightingale, 1982). Since primordial follicles cannot be regenerated (Hirshfield, 1991), chemical-induced depletion of this follicle pool can lead to infertility and premature ovarian failure (POF). A number of chemical classes can deplete follicles causing ovotoxicity, including the polycyclic aromatic hydrocarbon, 7,12-dimethylbenz[a]anthracene (DMBA) (Hoyer et al., 2009; Igawa et al., 2009; Mattison and Schulman, 1980). Since DMBA can induce DNA double strand break (DSBs) damage in oocytes and granulosa cells (Ganesan et al., 2013), there is a concern that exposure could lead to negative consequences for offspring should this damage be improperly repaired.

DSBs activate ataxia-telangiectasia mutated (ATM) protein which phosphorylates p53, a tumor suppressor protein. p53 can then activate DNA repair proteins, induce cell cycle arrest or initiate apoptosis if the DNA damage proves to be irreparable. Phosphorylated p53 activates a number of downstream pro-apoptotic molecules including *Bax* (Lane, 1992). BAX promotes apoptosis by binding to and antagonizing the action of BCL-2 protein, resulting in release of cytochrome c and activation of caspases to induce apoptosis (Weng et al., 2005). We and others have previously demonstrated that ovaries exposed to DMBA have increased levels of caspase 3 protein (Ganesan et al., 2013; Tsai-Turton et al., 2007). Additionally, BAX-deficient mice ovaries are resistant to DMBA-induced primordial follicle destruction (Matikainen et al., 2001).

Granulosa:granulosa and granulosa:oocyte cell to cell communication are necessary for maintenance of follicular viability. Communication between these cells occurs through gap junction intra-cellular channels, which directly connect the cytoplasmic compartments of neighboring cells and allow exchange of ions, metabolites and second messenger such as Ca^{2+} and inositol phosphates (Goldberg et al., 2004). Gap junctions are involved in regulation of cellular growth, metabolism and differentiation (Sohl and Willecke, 2003; Wei et al., 2004) and ovarian folliculogenesis (Simon et al., 2006). The major ovarian gap junction proteins are connexin (CX) 37 and 43 (Kidder and Mhawi, 2002a). CX37 communicates from the oocyte to granulosa cell

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(Simon et al., 1997), while CX43 functions in intra-granulosa cell communication (Granot et al., 2002; Kidder and Mhawi, 2002a). CX37 is thought to be involved in follicular development and ovulation as well as luteal tissue growth, differentiation, and regression (Borowczyk et al., 2006). *Cx37*-null mouse oocytes suffer growth retardation and do not survive to become meiotically competent (Carabatsos et al., 2000). Follicle growth is also interrupted: $Cx37^{-/-}$ granulosa cells form structures resembling corpora lutea in the absence of ovulation. CX43 levels are increased in granulosa cells following activation of follicular growth and maturation (Melton et al., 2001), while reduced granulosa cell expression of CX43 is linked to elevated apoptosis in porcine, bovine (Cheng et al., 2005; Johnson et al., 1999) and avian (Krysko et al., 2004) species. Thus, CX37 and CX43 play important roles in the ovary to maintain follicular and oocyte viability and quality.

Little is known about the impact of ovotoxicant exposures on ovarian function, thus in this study we investigated any impact of DMBA on ovarian gap junction genes *Cx37* and *Cx43* along with the pro-apoptotic cellular components, *p53* and *Bax*. We utilized a neonatal rat whole ovary culture method to determine the effect of a single DMBA exposure at two concentrations — 12.5 nM and 75 nM, since we have previously shown that these concentrations induce DNA damage and increased caspase 3 levels 8 days after exposure (Ganesan et al., 2013). Also, we have observed that these DMBA concentrations induced large primary and secondary follicle loss (unpublished data). Our hypothesis was that DMBA would alter *Cx37* and *Cx43* at time points prior to changes in pro-apoptotic genes (*p53* and *Bax*) and observed follicle loss supporting that they are targets of DMBA's mechanism of ovotoxicity.

Methods and materials

Reagents. 7,12-Dimethylbenz[a]anthracene (DMBA; CAS # 57-97-6), bovine serum albumin (BSA), ascorbic acid, transferrin, 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) 1× (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 TM SYBR Green PCR kit were purchased from Qiagen Inc (Valencia, CA). All primers were purchased from the Iowa State University DNA facility. All primary antibodies were purchased from Abcam (Cambridge, MA). RNA later was obtained from Ambion Inc. (Austin, TX). Goat anti-rabbit secondary antibodies were obtained from Santa Cruz Biotechnology, (Santa Cruz, CA). Ponceau S was from Fisher Scientific. ECL plus chemical luminescence detection kit was obtained from GE Healthcare, Amersham (Buckinghamshire, UK). Primers were obtained from the DNA facility of the Office of Biotechnology at Iowa State University (Ames, IA).

Ovary culture. Ovaries were collected from PND4 Fisher 344 female rats and cultured as described previously (Devine et al., 2002). Briefly, PND4 female F344 rat pups were euthanized by CO_2 inhalation followed by decapitation. Ovaries were removed, trimmed of oviduct and other excess tissues and placed onto 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 that had previously been equilibrated to 37 °C. A drop of medium was placed on top of each ovary to prevent it from drying. Ovaries were cultured for 4 days in control medium to allow development of large primary and secondary follicles, and were then treated once with medium containing vehicle control (1% DMSO) ± DMBA (12.5 nM or 75 nM) and the culture was maintained for four or eight days at 37 °C and 5% CO_2. These exposures have been found in our laboratory to induce large primary and/or secondary follicles after 8 days (unpublished data). Additionally, 75 nM DMBA was shown to cause primordial follicle loss after 15 days of exposure on alternate days, while the 12.5 nM exposure did not affect primordial follicle number (Igawa et al., 2009). Thus, the purpose of these experiments was to investigate the impact of DMBA exposures on large primary and secondary follicles. The medium was replaced every two days. One ovary per animal was placed in control medium, while the contralateral ovary was exposed to the experimental treatment.

RNA isolation and quantitative RT-PCR. RNA was isolated using an RNeasy Mini kit (Qiagen) and the concentration was determined using an ND-1000 Spectrophotometer (λ = 260/280 nm; NanoDrop technologies, Inc., Wilmington, DE) (n = 3; 10 ovaries per pool). Total RNA (200 ng) was reverse transcribed to cDNA utilizing the Superscript III One-Step RT-PCR (Qiagen). cDNA was diluted (1:20) in RNase-free water. Diluted cDNA (2 μl) were amplified on an Eppendorf PCR Master cycler using Quantitect SYBR Green PCR kit (Qiagen). Primers were designed by Primer 3 Input Version (0.4.0); *Cx37* forward — tgatcacaggtggttctgga; *Cx37* reverse — aggagaagtggggtgtgatg: *Cx43* forward — gagcgaggtttcaacagtgc; *Cx43* reverse — ccgaacacgacagcagttta: *Gapdh* forward — gtggacctcatggcctacat; *Gapdh* reverse — ggatgga attgtgagggaga: *p53* forward — tggtccagcaaatcctatc; *p53* reverse — gagtggaggaaatgggtcct: *Bax* forward — cgagctgatcagaaccatca; *Bax* reverse — ctcagcccatcttcttccag. The regular 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 20s at which point data were acquired. There was no difference in *Gapdh* mRNA expression between treatments, thus each sample was normalized to *Gapdh* before quantification. Quantification of fold-change in gene expression was performed using the $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen, 2001; Pfaffl, 2001). Expression level for control was set at 1, and treatment gene changes were expressed as fold-change relative to the vehicle control treated ovaries. Thus, fold-changes presented are increases above the control value of 1.

Protein isolation and Western blotting. Protein was isolated from cultured ovaries (n = 3; 10 ovaries per 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). Briefly, homogenized samples were placed on ice for 30 min, followed by two rounds of centrifugation at 10,000 rpm for 15 min and protein concentration was measured using a BCA protocol. Protein was stored at −80 °C until further use. SDS-PAGE was used to separate protein homogenates which were then transferred to a nitrocellulose membrane. Membranes were blocked for 1 h in 5% milk in Tris-buffered saline containing Tween 20, followed by incubation with anti-rabbit CX37 or CX43 antibody (1:100) for 36 h at 4 °C. Following three washes in TTBS (1X), membranes were incubated with species-specific secondary antibodies (1:2000) for 1 h at room temperature. Membranes were washed 3× in TTBS and incubated in chemiluminescence detection substrate (ECL plus) for 5 min followed by X-ray film exposure. Densitometry of the appropriate bands was performed using ImageJ software (NCBI). Equal protein loading was confirmed by Ponceau S staining of membranes and protein level was normalized to Ponceau S densitometry values.

Immunofluorescence staining. Ovaries were fixed in 4% paraformaldehyde for 2 h, transferred to 70% ethanol, embedded in paraffin, serially sectioned (5 μM thick), and every 10th section was mounted. Slides were deparaffinized in xylene and rehydrated with subsequent washes