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Estrogen receptor alpha overexpressing mouse antral follicles are sensitive to atresia induced by methoxychlor and its metabolites

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ABSTRACT

Methoxychlor (MXC) and its metabolites bind to estrogen receptors (ESRs) and increase ovarian atresia. To test whether ESR alpha (ESR1) overexpressing (ESR1 OE) antral follicles are more sensitive to atresia compared to controls, we cultured antral follicles with vehicle, MXC (1–100 μg/ml) or metabolites (0.1–10 μg/ml). Results indicate that MXC and its metabolites significantly increase atresia in ESR1 OE antral follicles at lower doses compared to controls. Activity of pro-apoptotic factor caspase-3/7 was significantly higher in ESR1 OE treated antral follicles compared to controls. ESR1 OE mice dosed with MXC 64 mg/kg/day had an increased percentage of atretic antral follicles compared to controls. Furthermore, pro-caspase-3 levels were found to be significantly lower in ESR1 OE ovaries than controls dosed with MXC 64 mg/kg/day. These data suggest that ESR1 OE ovaries are more sensitive to atresia induced by MXC and its metabolites *in vitro* and *in vivo* compared to controls.

1. Introduction

The ovaries of a mammalian female are composed of structural and functional units called follicles at different stages of maturity. The ovary is responsible for nurturing and facilitating the oocyte-containing follicle through various stages of its development until it can release the oocyte for fertilization [1–3]. However, by a mechanism that is largely unknown, a relatively small number of follicles are recruited from the initial pool to ovulate and release an oocyte, while the rest of the follicles undergo atresia [1,4]. Therefore, atresia is a process of programmed cell death (apoptosis) that occurs naturally in the granulosa cells of the ovary at all stages of follicular development throughout the reproductive lifespan. During follicular growth and development, approximately 99% of follicles undergo atresia due to apoptosis of granulosa cells and hence, atresia remains a prominent feature of ovarian function until all the follicles are exhausted, resulting in reproductive senescence [1,5,6]. Because the number of follicles endowed to the female at birth is finite, it is critical that sufficient numbers of healthy follicles be available for ovulation for normal reproduction [6,7]. However, environmental toxicants such as the organochlorine pesticide, methoxychlor (MXC), have been shown to increase antral follicle atresia in mouse ovaries [8–10]. MXC specifically targets antral follicles, thus, raising the concern that exposure to MXC could cause depletion of antral follicles in the ovary, leading to infertility and reproductive senescence [9,11,12]. Early reproductive senescence is a concern because it has been associated with an increased risk of chronic diseases such as osteoporosis and cardiovascular disease [13,14].

Previous studies have shown that MXC may induce ovarian toxicity *via* the estrogenic pathway [15,16]. The metabolites of MXC, mono-hydroxy MXC (MOH) and bis-hydroxy MXC (HPTE) are more potent estrogenic compounds than MXC and can inhibit antral follicle growth to a greater extent than MXC [17,18]. There is limited information available that explains whether ovaries with increased expression of estrogen receptors will be more sensitive to estrogenic chemicals such as MXC and its metabolites. Several studies have shown that estrogen receptor expression can be induced in tissues by exposure to estrogenic chemicals or by genetic polymorphisms [19–21]. In this study, we have analyzed whether ovaries with increased expression of estrogen receptors are more sensitive to MXC and its metabolites. Specifically, because MXC and its metabolites can bind to estrogen receptor alpha (ESR1) [22,23], we tested the effects of MXC and its metabolites on a transgenic mouse model in which estrogen receptor alpha (ESR1) was overexpressed (ESR1 OE) in the ovaries. We hypothesized that ovaries overexpressing ESR1 are more sensitive to antral follicle toxicity induced by MXC and its metabolites

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because of an increased number of binding sites for the estrogenic chemicals.

In a previous study, we reported that antral follicles of ESR1 OE were more sensitive to inhibition of growth induced by MXC and its metabolites *in vitro* compared to controls [24]. Inhibition of growth was observed with lower doses of MXC and its metabolites in ESR1 OE, but not in control antral follicles. Disruption in the normal ratio of ESR1:ESR2 in ESR1 OE mouse ovaries may be critical in driving the sensitivity of the antral follicle to MXC and its metabolites. Hence, in this study, we analyzed atresia in antral follicles of control and ESR1 OE mice treated with MXC and its metabolites to determine whether antral follicles of ESR1 OE mice are more sensitive to atresia induced by lower doses of the chemicals compared to controls. To compare follicular atresia in both genotypes at the molecular level, we also evaluated the levels of key players in the apoptotic pathway, including the anti-apoptotic factor, B-cell lymphoma/leukemia protein-2 (Bcl-2) and the pro-apoptotic factors, Bcl-2 associated X protein (Bax), Bcl-2 interacting domain (Bid) and cysteine aspartate-specific protease – 3 (caspase-3).

2. Materials and methods

2.1. Chemicals

MXC (99%) was purchased from Chemservice (West Chester, PA). For *in vivo* experiments, mice were dosed with 8, 16, 32 and 64 mg/kg/day. Four stock solutions of MXC were prepared by using sesame oil (SES; Thermo Fisher Scientific Inc., Rockford, IL) as the vehicle. The stock concentrations were 5 mg/ml for 8 mg/kg/day dose, 10 mg/ml for 16 mg/kg/day dose, 20 mg/ml for 32 mg/kg/day and 40 mg/ml for 64 mg/kg/day dose. To keep the doses constant, mice were administered 1.6 ml/kg of the stock solutions.

For *in vitro* experiments, stock solutions of MXC, MOH and HPTE were prepared by dissolving them in dimethylsulfoxide (DMSO) (Sigma, St. Louis, MO). MOH and HPTE (99%) were synthesized in Dr. Vincent Njar's laboratory (University of Maryland, Baltimore now at Thomas Jefferson University, Philadelphia, PA). MXC stock solutions were prepared by dissolving 1.33, 13.3 and 133 mg/ml resulting in a final concentration of 1, 10 and 100 μg/ml per well. MOH and HPTE stock solutions were prepared by dissolving 0.133, 1.3 and 13.3 mg/ml resulting in a final concentration of 0.1, 1 and 10 μg/ml per well. The doses selected for the *in vitro* and *in vivo* studies were based on previously published studies showing that these concentrations cause increased antral follicle atresia and inhibition of antral follicle growth in CD-1 mice [8–11].

2.2. Animals

ESR1 OE and control mice (32–39 day old cycling females) that were used in this study were generated using C57BL6 and FVB mice as described previously [24,25]. ESR1 OE mice were validated for the overexpression of ESR1 gene and protein levels in the ovaries by quantitative real-time polymerase chain reaction (qPCR) and immunohistochemical staining techniques [24].

The mice were housed in the University of Illinois core animal facility under a 12:12 dark:light cycle and provided food and water *ad libitum*. The University of Illinois Institutional Animal Care and Use Committee approved all animal procedures used in this study.

2.3. In vivo dosing regimen

ESR1 OE and control mice were administered either sesame oil or MXC intraperitoneally using a 1 ml syringe at 1.6 ml/kg body weight. The doses were adjusted based on the animal's most recent body weight. The mice were dosed continuously for a period of 20 days, as previous studies have shown that this length of exposure does not cause overt toxicity, but does induce antral follicle atresia [9]. In addition, to be consistent with previous studies and to minimize variability in results, intraperitoneal route of administration was used. After dosing, all samples were collected when the mice were in estrus to decrease variability at various days of the estrous cycle. Moreover, previous studies have shown that *in vivo* dosing with MXC causes persistent estrus [9,11].

2.4. Antral follicle culture

Antral follicles (determined by appearance and relative size) were isolated from ESR1 OE and control ovaries using fine watchmaker forceps. About 75–80 antral follicles (300–400 μm) were obtained from at least two mice of each genotype per experiment. The follicles were then randomly divided into five groups – non-treatment (NT), vehicle-control (DMSO) and three chemical treatments of MXC, MOH or HPTE. Increasing concentrations (1.33, 13.3 and 133 mg/ml) of MXC and (0.133, 1.33 and 13.3 mg/ml) of MOH and HPTE were made to allow an equal volume to be added to each of the treatment groups in the 96-well culture plate to control the solvent concentration. The final concentrations of MXC in each well of the culture were 1, 10 and 100 μg/ml. Similarly, final concentrations of MOH and HPTE in culture were 0.1, 1, and 10 μg/ml. MOH and HPTE were used at 10-fold lower concentrations than MXC because the metabolites of MXC are thought to be more toxic than the parent compound [10]. Moreover, from a physiological stance, a considerable quantity of MXC is likely broken down to its metabolites by the liver before it reaches the ovaries. In the vehicle treatment group, DMSO was used at 0.075%, which is not toxic to cultured follicles [8,10]. The doses selected for *in vitro* studies were based on previously published studies showing that these concentrations of MXC, MOH and HPTE induce toxicity in antral follicles and granulosa cell culture models [8,10]. A non-treatment group was included to control for culture conditions and follicles in this group were placed only in supplemented α-MEM devoid of either DMSO or chemicals.

The follicles were cultured in supplemented α-MEM as described previously [8,10]. Briefly, supplemented α-MEM was prepared using 1% ITS (10 ng/ml insulin, 5.5 ng/ml transferrin, 5.5 ng/ml selenium), 100 U/ml penicillin, 100 mg/ml streptomycin, 5 IU/ml human recombinant follicle-stimulating hormone (FSH; Dr. A.F. Parlow, National Hormone and Peptide Program, Harbor-UCLA Medical Center, Torrance, CA), and 5% fetal calf serum (Atlanta Biologicals, Lawrenceville, GA). Follicles were incubated for 96 h at 37 °C in 95% air and 5% CO_2.

2.5. Histological evaluation of atresia

For the *in vivo* study, the ovaries of the mice in estrus were collected and fixed in Dietrick's fixative. After fixation, the ovaries were dehydrated and embedded in Paraplast (VWR scientific, West Chester, PA), serially sectioned at 8 μm and stained with Weigert's hematoxylin–picric acid methyl blue. Follicles were classified as antral if they contained five or more layers of granulosa cells and a clearly defined antral space. Antral follicles were classified as atretic if 10% of the granulosa cells were apoptotic (defined by the presence of pyknotic or apoptotic bodies in the granulosa cell layer), the granulosa cells were disorganized or the oocyte was fragmented or degenerating.

For evaluation of atresia in antral follicle cultures, the antral follicles exposed to DMSO or MXC/MOH/HPTE were fixed for at least 24 h in Dietrick's fixative at the end of the culture. After a series of washes, the follicles were embedded in plastic (Technovit 7100), sectioned at 2 μm, and stained using Lee's methylene blue – basic fuchsin. The amount of follicular atresia was examined in each antral follicle by the presence of pyknotic bodies. Follicles were scored as healthy or atretic using a scale of 1–4 (1 = healthy follicle; 2 = 10% apoptotic bodies/per follicle meaning an early atretic follicle; 3 = 11–30% of apoptotic bodies/per follicle meaning a mid atretic follicle; 4 = greater than 30% apoptotic bodies/per follicle meaning a late atretic follicle) as previously described [8,18]. All scoring was done without knowledge of a genotype or treatment group. At least 2–3 antral follicles from three separate cultures were analyzed for atresia from each treatment group.

2.6. Caspase activity

Caspase activity levels were analyzed in antral follicles that were exposed to DMSO or MXC *in vitro* using the Caspase-Glo 3/7 Assay (Promega Corporation, Madison, WI). The Caspase-Glo 3/7 Assay is a homogeneous, luminescent assay that measures caspase-3 and -7 activities. Antral follicles were cultured with DMSO or MXC as described earlier. At the end of 96 h, the antral follicle from each well and 100 μl of the supplemented media were placed into a white 96-well plate. As per manufacturer's protocol, the reagents in the assay kit were mixed and added into each well containing the antral follicle in the supplemented media. The addition of the reagents in the assay kit results in cell lysis of the antral follicle, followed by the generation of a luminescent signal produced by luciferase. Luminescence is proportional to the amount of caspase activity present and was measured using a microplate reader with chemiluminescent filters.

2.7. Western blotting

Protein analyses of pro-caspase-3 and PARP1 were carried out using ovaries from control and ESR1 OE mice that were treated with vehicle or MXC *in vivo*. Antral follicles from an ovary were isolated and homogenized using 250 μl T-Per (Thermo Fisher Scientific, Rockford, IL) containing protease inhibitors (Roche Diagnostics, Mannheim, Germany). After homogenization, the protein concentration in the lysate was determined by using a bicinchoninic acid (BCA) assay kit (Thermo Fisher Scientific, Rockford, IL). Electrophoresis and immunoblotting were performed using XCell SureLock Mini-Cell Blot Module Kit and recommended reagents as per manufacturer's protocol (Invitrogen, Carlsbad, CA). The protein lysate (3–5 μg) was loaded on precast 4–12% bis-tris sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE; Invitrogen, Carlsbad, CA), followed by wet transfer of the proteins to a blot at 4 °C. As each precast gel has 17 wells/gel, we loaded ESR1 OE and control samples on separate gels. However, all treatment groups (5 treatments; $n = 3$) of each genotype were loaded and run on the same gel (Fig. 5A right panel). The blots were incubated overnight at 4 °C with primary antibodies, anti-pro-caspase-3 and anti-PARP1 (1:1000; Cell Signaling Technologies, Danvers, MA), followed by incubation with