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Environmental (anti-)androgenic chemicals affect germinal vesicle breakdown (GVBD) of *Xenopus laevis* oocytes *in vitro*

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ABSTRACT

Progesterone-induced germinal vesicle breakdown (GVBD) of *Xenopus* oocytes *in vitro* was used to study endocrine disrupting activity of chemicals in previous studies. In this study, we investigated for the first time effects of environmental androgens on oocyte maturation and effects of anti-androgens on androgen-induced oocyte maturation, using *Xenopus* GVBD *in vitro*. Trenbolone and nandrolone, two environmental androgens, were found to induce *Xenopus* GVBD at low concentrations. The potential of trenbolone to induce GVBD was approximately 100-fold lower than that of testosterone, while nandrolone had a several-fold lower potential than testosterone. Our findings have aroused new concerns for effects of environmental androgens on amphibian oocyte maturation at environmentally relevant concentrations, and suggested that *Xenopus* GVBD can be used to test androgenic activity of suspicious environmental androgens. Androgen receptor (AR) antagonist flutamide at 10 μM only exhibited a weakly inhibitory effect on androgen-induced GVBD, while another known AR antagonist vinclozolin had no effect even at high concentrations. The results show that *Xenopus* GVBD is not sensitive to AR-mediated environmental anti-androgens. In contrast to flutamide and vinclozolin, methoxychlor (a weaker AR antagonist) inhibited dramatically androgen-induced GVBD, suggesting that androgen-induced *Xenopus* GVBD can be used to study non-AR-mediated effects of chemicals on oocyte maturation.

1. Introduction

Xenopus laevis oocytes have been used for many decades to study steroid-induced maturation due to large size and relative abundance (Hammes, 2004). Historically, progesterone was considered as the physiological mediator of *Xenopus* oocyte maturation, which is accompanied by germinal vesicle breakdown (GVBD), spindle formation, and extrusion of the first polar body (Bayaa et al., 2000; Ferrell, 1999). However, recent observations have proven that androgens rather than progesterone are the physiologic regulators of oocyte maturation. *In vitro* experiments have shown testosterone as well as progesterone can induce GVBD of *Xenopus* oocytes, and effective concentrations of testosterone (~50 nM) are lower than those of progesterone (submicromolar) (Goascogne et al., 1985; Lutz et al., 2001, 2003). *In vivo* experiments, a dramatic increase in androgen levels but not in progesterone levels was found in serum of female frogs with the injection of human chorionic gonadotropin (HCG), which can induce oocyte maturation and ovulation (Lutz et al., 2001). Furthermore, it has been demonstrated that androgen-induced *Xenopus* oocyte maturation is mediated by classical androgen receptors (ARs), and AR antagonist flutamide can inhibit androgen-triggered maturation (Lutz et al., 2001, 2003).

Eco-toxicological effects and human health risk of endocrine-disrupting chemicals (EDCs), such as environmental (anti-)estrogens and (anti-)androgens, have attracted more attention for several decades (Meeker, 2012; Schug et al., 2011; Söffker and Tyler, 2012; Vandenberg et al., 2012). Amphibians are susceptible to EDCs due to their permeable skins, biphasic life cycle and some biological characteristics (Van der Schalie et al., 1999). Numerous studies have described effects of EDCs on gonadal differentiation, the secondary sex characteristic development, and metamorphic process of amphibians (Crump et al., 2002; Hayes et al., 2002, 2010; Porter et al., 2011; Qin et al., 2003). Also, some of EDCs, such as ethinyl estradiol, atrazine, 2,4-dichlorophenoxyacetic acid and methoxychlor, have been shown to affect oocyte maturation in amphibians, suggesting potential adverse effects on reproductive function of amphibians (Fort et al., 2002; Ghodageri and Katti, 2013; Pickford and Morris, 1999; Stebbins-Boaz et al., 2004). Given that androgens can induce oocyte maturation and anti-androgen flutamide can inhibit androgen-induced oocyte maturation, we propose that environmental androgens and anti-androgens could disrupt oocyte maturation of amphibians.

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Androgenic-anabolic steroids (AAS) used in human and animals are released into the environment and become environmental androgens. Trenbolone, one of AAS, is widely used as a growth promoter in animal agriculture. Some studies investigated the levels of trenbolone in wastewater, soil and solid waste in feedlots (Khan et al., 2008; Qu et al., 2012). Trenbolone was also found in the receiving environment of feedlot wastewater and general environment (Gall et al., 2011; Khan and Lee, 2012; Liu et al., 2011). Generally, the concentrations of trenbolone in water are at the level of ng/L (Liu et al., 2012), but the μg/L level of trenbolone was also reported in some water samples (Khan and Lee, 2012). Nandrolone as a doping agent is widely abused to improve athletic performance and body image (Hemmersbach and Große, 2010; Lumia and McGinnis, 2010). Nandrolone is also used medically as a treatment for anemia and other diseases (Cederholm and Hedström, 2005; Deicher and Hörl, 2005). Several studies reported the presence of nandrolone in wastewater, and even the level exceeded 60 ng/L (Backe et al., 2011; Sun et al., 2010). Recently, two studies described effects of trenbolone on the growth, survival, and sexual differentiation of *X. laevis* (Olmstead et al., 2012; Finch et al., 2013). Overall, available information on adverse effects of AAS on amphibians has been very limited.

In addition, some chemicals have been demonstrated to exhibit AR-mediated anti-androgenic activity. Several fungicides, such as vinclozolin, procymidone, and prochloraz, are well-known AR antagonists (Aït-Aïssa et al., 2010; Kolle et al., 2011; Wilson et al., 2008). Methoxychlor was shown to have a very weak AR-antagonistic activity (Maness et al., 1998). However, whether these environmental anti-androgens affect androgen-mediated oocyte maturation of amphibians has never been studied. In this study, our aim was to investigate effects of environmental androgens (trenbolone and nandrolone) on amphibian oocyte maturation, and effects of environmental anti-androgens (vinclozolin and methoxyclor) on androgen-induced oocyte maturation using *Xenopus* GVBD *in vitro*.

2. Materials and methods

2.1. Chemicals

Testosterone (99.5%), trenbolone (98.0%), nandrolone (99.0%) and vinclozolin (99.0%) were purchased from Dr. Ehrenstorfer (Germany). Methoxychlor (98.9%) was purchased from Accustandard (USA). Flutamide (96.0%) was purchased from ChromaDex (USA). Dimethyl sulfoxide (DMSO) and 3-aminobenzoic acid ethyl ester (MS-222) were from Sigma–Aldrich (USA). All chemicals above were dissolved in DMSO and stored at 4 °C. Collagenase I was purchased from Gibco (USA). Trichloroacetic acid, sodium pyruvate, and 4-(2-hydroxyethyl)-1-piperazineethanesulphonic-acid (HEPES) were purchased from Biotopped (China). Commercial amphibian diet was obtained from Totoro Supplies (HongKong, China). Other reagents were purchased from Beijing Chemical Reagents (Beijing, China).

2.2. Experimental animals

Adult female *X. laevis* frogs (three years old) were raised in glass tanks in charcoal-dechlorinated water at 22 ± 2 °C. The water quality was as follows: chlorine concentration <5 μg/L, iodine concentration 2.14–3.92 μg/L, pH 6.5–7.0, the dissolved oxygen concentration >5 mg/L, and water hardness ($CaCO_3$) approximately 150 mg/L. The water was changed completely twice weekly after feeding with chopped pork liver: commercial amphibian diet (1:1). Fluorescent lighting provided a photoperiod of 12 h light: 12 h dark with a light intensity ranging from 100 to 200 lux at the water surface. Each frog was injected by 100 IU human chorionic gonadotropin (HCG) two weeks before surgery. Female frogs were anaesthetized by submersion in 100 mg/L MS-222 buffered with 200 mg/L of sodium bicarbonate about 15 min until their reflexes disappeared. In every independent experiment, the female frog was placed on an ice bed and 2–4 ovary lobes were pulled out carefully through a small ventral incision (0.8–1 cm). Then, the incision was closed in two layers with absorbable gut for the muscle layer and nylon suture for the skin closure. Finally, the frog was put in the water. *Xenopus* husbandry and all animal procedures complied with the *Xenopus* guidline edited by Green (2010), and all animal procedures also accorded with Regulations for the Administration of Affairs Concerning Experimental Animals (State Science and Technology Commission of the People's Republic of China, 1988).

2.3. Preparation and culture of Xenopus oocytes

Ovary lobes were cut into small strips, and rinsed several times in OR-2 solution (NaCl 82.5 mM, KCl 2.5 mM, $MgCl_2$ 1.0 mM, HEPES 5.0 mM, pH 7.6), then digested by 0.15% (w/v) collagenase I in OR-2 for 2 h at a shaker (TY10QB-128, Kylin-Bell, China) until blood vessels and follicle cell layers were removed from the oocytes. The oocytes were rinsed 4–5 times in fresh OR-2 and ND-96 solution (NaCl 96.0 mM, KCl 2.0 mM, $CaCl_2$ 1.8 mM, $MgCl_2$ 1.0 mM, HEPES 5.0 mM, sodium pyruvate 2 mM, pH 7.6), respectively. Then, the oocytes were transferred to a disposable 100-mm petridish containing fresh ND-96 solution. Follicle cell-free oocytes at stage V–VI (diameter 1.2–1.3 mm) were picked out using Pasteur pipette under a stereomicroscope for experiments.

2.4. GVBD assay for testosterone, trenbolone, nandrolone

The oocytes at stage V–VI were plated (20/well) in 12-well polystyrene culture plates (Costar Corporation, Cambridge, MA) with 2 mL ND-96 solution containing series of concentrations (10 nM–10 μM) of testosterone, trenbolone, and nandrolone. Each treatment group consisted of three replicate wells. DMSO concentration was 0.01% (v/v) in the solvent control. The oocytes were then incubated in an incubator (MIR-153, Sanyo, Japan) at 22 °C. After 4h-incubation, GVBD was determined visually by the emergence of a white Roux spot at the animal pole under a stereomicroscope (Pickford and Morris, 1999). Culture plates were replaced into the incubator for a prolonged 4h-incubation. At the end of the whole 8h-incubation, the oocytes were fixed in 5% (w/v) trichloroacetic acid, and GVBD was verified by breakdown of germinal vesicles in opened oocytes (Pickford and Morris, 1999). The GVBD percentage was calculated by counting the number of mature oocytes of 20 oocytes in each well. The experiment for each test compound was repeated three times using oocytes derived from different *X. laevis*.

2.5. GVBD assay for anti-androgenic compounds

According to the method described above, the oocytes were exposed to series of concentrations of flutamide or vinclozolin or methoxyclor in the presence or absence of 10 nM testosterone. After 4h-incubation, GVBD was determined visually by the emergence of a white Roux spot and verified by breakdown of germinal vesicles in trichloroacetic acid-fixed oocytes. The GVBD percentages was calculated by counting the number of mature oocytes of 20 oocytes in each well. The experiment for each test chemical was repeated two times using oocytes derived from different *X. laevis*.