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Atrazine suppresses FSH-induced steroidogenesis and LH-dependent expression of ovulatory genes through PDE-cAMP signaling pathway in human cumulus granulosa cells

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ABSTRACT

Atrazine (ATR) alters female reproductive functions in different animal species. Here, we analyzed whether ATR disturbs steroidogenic and ovulatory processes in hormone-stimulated human cumulus granulosa cells and mechanism of its action. Results showed that treatment of human cumulus granulosa cells with 20 μM ATR for 48 h resulted in lower FSH-stimulated estradiol and progesterone production. ATR reduced mRNA levels of aromatase (*CYP19A1*), steroidogenic acute regulatory protein (*STAR*) and luteinizing hormone/choriogonadotropin receptor (*LHCGR*). Addition of hCG 48 h after FSH and ATR treatment did not trigger maximal expression of the ovulatory genes amphiregulin (*AREG*) and epiregulin (*EREG*). Mechanistic experiments showed that ATR activated cPDE and decreased cAMP level. Addition of total PDE and specific PDE4 inhibitors, IBMX and rolipram, prevented ATR's action on *CYP19A1* and *STAR* mRNA expression in FSH-stimulated human cumulus granulosa cells. This study suggests that ATR alters steroidogenesis and ovulatory process in human cumulus granulosa cells jeopardizing female reproduction.

1. Introduction

Atrazine (2-chloro-4-ethylamino-6-isopropylamino)-s-triazine (ATR) is a common herbicide and environmental contaminant with the ability to disrupt the endocrine and the reproductive system. ATR was banned in the EU countries in 2004 (Sass and Colangelo, 2006); however, it is still applied in the United States, Switzerland, China and certain countries of Africa and Asia for agricultural and residential purposes. It is worth mentioning that ATR is detected in the soil, groundwater and drinking water in the countries where it has been banned (Nödler et al., 2013; Vonberg et al., 2014a, 2014b). Human exposure to ATR occurs through contaminated agricultural land, surface waters and groundwaters (Barr et al., 2007), as evident by its presence in the urine of pregnant women (Chevrier et al., 2011) or in breast milk in concentration of 0.02 mg/kg (Balduini et al., 2003). Moreover, epidemiological studies shown that women who lived in rural area where ATR is used extensively (Illinois) have menstrual cycle length irregularity with longer follicular phases and decreased levels of estradiol and progesterone metabolites (Cragin et al., 2011). Also, it has been demonstrated that presence of ATR in maternal urine samples was associated with fetal growth impairment and small head circumference (Chevrier et al., 2011).

Animal *in vivo* and *in vitro* studies are widely used and the results obtained in these studies are often the first indicators of potential reproductive or developmental effects of chemicals. Such studies have already provided important information regarding ATR's toxic effect on the normal development and the function of reproductive organs in different species (Cooper et al., 2000; Kniewald et al., 2000; Stoker et al., 2000; Hayes et al., 2002, 2011; Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010; Kucka et al., 2012). In the follicle-stimulating hormone (FSH)-stimulated rat granulosa cells, ATR decreases estradiol level and expression of aromatase (*Cyp19a1*). Concurrently, ATR induces over-expression of luteal markers, such as steroidogenic acute regulatory protein (*Star*) and cytochrome P450 side-chain cleavage enzyme (*Cyp11a1*),

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followed by an increase in the progesterone synthesis (Pogrmic-Majkic et al., 2014). In addition, ATR selectively decreases the luteinizing hormone (LH) receptor (*Lhr*) mRNA levels, thus causing low expression of the ovulatory genes upon human chorionic gonadotropin (hCG) stimulation (Fa et al., 2013). Reproductive toxicity and anti-ovulatory action of ATR has been demonstrated in whole animal studies. Gestational exposure to high doses of ATR reduces the body weight of the offspring and causes the delay in vaginal opening (Davis et al., 2011). High doses of ATR administered orally to adult female rats have been reported to reduce the LH surge and the length of the estrous cycle (Foradori et al., 2009) and decrease the number of corpora lutea and oocytes released from the ovaries (Foradori et al., 2014). Moreover, ATR administered to immature female rats suppressed the pregnant mare serum gonadotropin-stimulated *Lhr* and *Cyp19a1* mRNA expression and estradiol secretion in ovarian granulosa cells, thus blocking ovulation (Samardzija et al., 2016).

Studies using animal models also provide an important insight into the mechanism by which ATR affects the steroidogenic process. ATR interferes with the turnover of cyclic adenosine monophosphate (cAMP) by inhibiting the cAMP phosphodiesterase (cPDE) activity and by increasing the cAMP level. ATR was reported to act as a competitive inhibitor of cPDE derived from bovine hearts (Roberge et al., 2004). In rat granulosa, Leydig and pituitary cells, ATR acts primarily through the cPDE/cAMP signaling pathway (Pogrmic et al., 2009; Pogrmic-Majkic et al., 2010, 2014; Kucka et al., 2012; Karmaus and Zacharewski, 2015; Pogrmic-Majkic et al., 2016). Besides cAMP, many other pathways are involved in ATR action, such as extracellular signal-regulated protein kinases 1 and 2 (ERK1/2) in rat Leydig and granulosa cells (Fa et al., 2013; Karmaus and Zacharewski, 2015), reactive oxygen species (ROS), calcium, protein kinase C (PKC), and the epidermal growth factor (EGFR) in rat Leydig cells (Pogrmic-Majkic et al., 2016), protein kinase B (AKT) in rat granulosa cells (Pogrmic-Majkic et al., 2014) and human choriocarcinoma cell line (JEG3) (Suzawa and Ingraham, 2008), G protein estrogen receptor in different cancer cells (Albanito et al., 2008), and nuclear receptor 5A subfamily in JEG3 cells (Suzawa and Ingraham, 2008).

While the effect of atrazine on granulosa cells' steroidogenesis in animals is well studied, the effect of this herbicide in human granulosa cells remains largely unknown. Data on the human adrenocortical carcinoma H295R cells and the human placental carcinoma cell line JEG3 show that ATR elicits the estrogen action by up-regulating the aromatase activity (Sanderson et al., 2000, 2001; Heneweer, 2004; Fan et al., 2007a, 2007b); however, similar responses in the human breast cancer cell line MCF-7 (Sanderson et al., 2001) or the human ovarian granulosa-like tumor KGN cell line (Morinaga et al., 2004) were not replicated.

Since the dose of 20 μM ATR has been shown to alter steroidogenic process in animal granulosa cells during short exposure period (Fa et al., 2013; Pogrmic-Majkic et al., 2014; Wirbisky and Freeman, 2015), we aimed to determine whether this dose of ATR disturbs human cumulus granulosa cells' steroidogenic function and to identify possible underlying molecular mechanism(s). We have placed the focus on the enzymes that are affected by ATR exposure in rat granulosa cells: CYP19A1, involved in estradiol synthesis, STAR and CYP11A1, involved in progesterone production, and the luteinizing hormone/choriogonadotropin receptor (LHCGR), responsible for the ovulatory response. Here, we show that ATR diminishes the steroidogenic process in human cumulus granulosa cells by inhibiting the FSH-induced *CYP19A1* mRNA expression and estradiol production, as well as *STAR* mRNA expression and progesterone production. ATR also diminishes the FSH-induced *LHCGR* mRNA expression, resulting in the reduced capability of hCG to induce expression of the ovulatory genes, such as amphiregulin (*AREG*) and epiregulin (*EREG*). Moreover, the results of this study highlight that ATR's anti-steroidogenic action is mediated through activation of the cPDEs.

2. Material and methods

2.1. Chemicals

Atrazine, Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham with L-glutamine, 15 mM HEPES (DMEM/F12), BSA fraction V, penicillin, streptomycin, fibronectin, 8-bromo-adenosine 3′5′-cyclic monophosphate (8-Br-cAMP), forskolin, rolipram, 3-isobutyl-1-methylxanthine (IBMX), and TRIzol Reagent were obtained from Sigma-Aldrich Company (Germany). Purified recombinant hFSH was from Serono (Randolph, MA, USA), hCG (Pregnyl, 3000 IU/mg) was from Organon (West Orange, NJ, USA). The cAMP EIA Kit was purchased from Cayman Chemicals (Ann Arbor, MI, USA) and PDE activity assay kit (Colorimetric) from Abcam (Cambridge, UK).

2.2. Human cumulus granulosa cell culture and treatments

Human cumulus granulosa cells were obtained from women undergoing *in vitro* fertilization (IVF) at the Clinic for Gynecology and Obstetrics, Clinical Center of Vojvodina, Novi Sad, Serbia. The study was approved by the Ethics Committee of the Clinical Center of Vojvodina (approval number: 00–15/271) and the signed informed consent was obtained from each participant. Patient information was not collected for the purpose of this study. Human cumulus granulosa cells were used as an experimental model since their response to FSH resembles differentiation of the preantral to the preovulatory granulosa cells (Baumgarten et al., 2014). We used the protocol for obtaining and isolation of human cumulus granulosa cells as previously published (Baumgarten et al., 2014). Briefly, after controlled ovarian stimulation, large follicles were aspirated from the patients undergoing IVF. The cumulus-oocyte-complexes were then removed from the follicular aspirates and the cumulus cells were mechanically separated from the oocyte. Depending on the experimental design, the cumulus cells from all follicles of a single patient or the cells obtained from more than one patient were pooled together and used for the subsequent analyses, as described in figure legends. The cells were centrifuged at 1000 × g for 5 min, resuspended in DMEM/F12 1:1 (vol:vol) containing 100 IU/mL penicillin, 0.1 mg/mL streptomycin, and 0.1% BSA and plated in 24-well plates (0.1×10^6 cells/well) that had been pre-coated with fibronectin. One day after plating, the cells were treated with 20 μM ATR. To analyze the effect of ATR exposure on progesterone and estradiol levels and *STAR*, *CYP19A1* and *LHCGR* mRNA expression, human cumulus granulosa cells were stimulated with 100 ng/mL FSH in the presence or absence of 20 μM ATR for 48 h. To analyze the human cumulus granulosa cells' response to the ovulatory action of hCG, the cells were treated with 100 ng/mL FSH in the presence or absence of 20 μM ATR for 48 h. Afterwards, the cells were stimulated with 1 IU hCG for 3 h. In some experiments, the cells were either stimulated with forskolin (1 μM) or 8-Br-cAMP (1 mM) in the presence or absence of 20 μM ATR for 48 h or pretreated with the inhibitors of PDEs IBMX (1 mM) and rolipram (10 μM) for 1 h and then treated with 100 ng/mL FSH in the presence or absence of 20 μM ATR for 48 h, followed by assessment of *STAR* and *CYP19A1* mRNA expression. For cAMP measurements and PDE activity assay, the cells were treated with 100 ng/mL FSH in the presence or absence of 20 μM ATR for 1 h.

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